Serine Palmitoyltransferase and Ceramide Kinase in Embryo Development of Loblolly Pine

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Serine Palmitoyltransferase and Ceramide Kinase in Embryo Development of Loblolly Pine

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LIST OF SYMBOLS AND ABBREVIATIONS

a. a.	amino acids
ABA	abscisic acid
BAP	benzylaminopurine
C1P	ceramide 1 phosphate
CERK	ceramide kinase
ER	endoplasmic reticulum
EST	expressed sequence tags
GCS	glucosylceramide synthase
GlcCer	glucosylceramide
Hcerk	ceramide kinase
IPC	inositolphosphorylceramide
LCB	sphingosine long chain base
LP	Loblolly pine
LPP	lipid phosphate phosphatase
LSCs	liquid suspension cultures
MAPKKK	mitogen activated protein kinase kinase kinase
NAA	a-naphthalene acetic acid
nts	nucleotides
PCD	programmed cell death
PH	pleckstrim homology
Pt	putative
RT	room temperature
S1P	sphingosine 1-phosphate
SE	somatic embryogenesis
SM	sphingomyelin
SMS	sphingomyelin synthase
SPT	serine palmitoyltransferase
SPP-1	S1P phosphatase-1
SphK	sphingosine kinase

TMD	transmembrane domains
3-KDS	3-ketodihydrosphinganine
2, 4 D	dichlorophenoxyacetic acid
WPM	woody plant medium

SUMMARY

Using the known sequences for serine palmitoyltransferase (SPT) and ceramide kinase (CERK) from Arabidopsis, candidates for the corresponding genes in Loblolly pine were cloned and examined during embryogenesis.

The cloned two cDNA sequences from Loblolly pine, which has similarity of 81% and 88% respectively to two subunits of SPT1 and SPT2 in Arabidopsis, were presumed as the Loblolly pine SPT1 and SPT2 (Pt-SPT1 and Pt-SPT2). A few different versions of Pt-SPT1 mRNAs (2223 nts, 756 nts, 822 nts, and 754 nts respectively), most likely the alternative splicing results, were found. Three of these mRNAs are capable of encoding proteins. The long version (2223 nts) encodes a protein with 484 amino acids (Pt-SPT1); two short versions (822 nts, 756 nts) encode a 90 a.a. protein. Another cDNA sequence of 2396 nts encodes a protein of 493 a.a. (Pt-SPT2). Both predicted Pt-SPT1 and Pt-SPT2 proteins possess highly conserved serine palmitoyltransferase functional domains (E value $5.7e^{-61}$). Their expression patterns are different between somatic and zygotic embryogenesis.

Two different versions of mRNAs, with 2786 nts (long), and 2320 nts (short) respectively, of ceramide kinases in Loblolly pine (Pt-CERKs) have been cloned. The long version encodes a protein with 721 a.a.; the short version with 560 a.a. The expression patterns for these two CERK mRNAs are different during embryo development. The long version is constitutively expressed, while the short one is only expressed in some stages with much lower expression level. Overexpression Pt-CERKL, Pt-CERKS, and Pt-CERKF in *E.coli* and function analysis *in vitro* show that all Pt-

CERKs appear to have the same catalytic functions as their homologs in human and Arabidopsis, but with different efficiency. The catalytic efficiency was dramatically lower in the short Pt-CERK protein compared with the long Pt-CERK protein and Pt-CERKF. The membrane system is not necessary for the catalytic reactions of these three Pt-CERKs *in vitro* and Pt-CERKs were less dependent on the Ca^{2+} ions.

Thus, these studies have provided the first information about SPT- and CERK- like proteins in loblolly pine, and open new avenues of investigation for the roles of sphingolipids in embryonic development.

Chapter 1: Introduction

Loblolly pine (LP) is an economically important tree species which provides raw materials for building, pulp and paper industry, energy, and a renewable biomass for biofuels in the United States. Breeding elite strains of Loblolly pine is critical to supply wood and biomass for the forest product industry and bio-energy. Propagation of valuable Loblolly pine from genetically improved strains through embryogenesis has been practiced for many years and great progress has been achieved. However, some factors still limit its application, for example, embryos aborting before mature, abnormal embryo formation, embryo culture decline over time, and mature embryo with low germination rate and weak somatic seedlings (Pullman et al. 2004). To solve these problems, more molecular studies of embryo development are necessary (Ciavatta et al. 2001).

Sphingolipids are important cell membrane components, and have been reported to function as important signal molecules in cell growth, cell death, and are involved in embryogenesis in animals (Bird & Kimber 1984; Kudo et al. 2004). Some sphingolipids work as the makers in cell-cell interactions during embryogenesis, differentiation, and neuron-developmental processes (Bird & Kimber 1984; Kudo et al. 2004). In invertebrates, the absence of serine palmitoyltransferase (SPT) activity leads to embryonic lethality (Adachi-Yanada et al. 1999). SPT is a key enzyme in sphingolipid biosyntheses and controls sphingolipid levels by influencing ceramide level inside cells (Snell et al. 1970; Williams et al. 1984). As the precursor of all sphingolipids, ceramide is known to mediate cell cycle arrest and to activate cell apoptotic pathway (Obeid et al. 1993; Perry & Hannun 1998). Another interesting sphingolipid metabolic enzyme, ceramide kinase, can phosphorylate ceramide to generate ceramide 1 phosphate (C1P), which has mitogenic effects (Gijsbers et al. 1999). Therefore,

ceramide kinase is one of the conversion points in controlling ceramide level and in changing cell fate (from cell death to cell survival). So far, there are no literature reports about sphingolipids in pine. Therefore, I have chosen to investigate these two enzymes in Loblolly pine.

Since somatic embryogenesis is a process in which the embryo develops under abnormal circumstances, it motivated the present study to investigate the function of serine palmitoyltransferase and ceramide kinase during Loblolly pine embryo development. Accordingly, this study has been conducted to understand if serine palmitoyltransferase and ceramide kinase change during embryo development of Loblolly pine. First, total mRNA was isolated from zygotic embryos of Loblolly pine and then converted into cDNAs using RACE kit. Gene specific primers were designed to clone SPT and CERK in Loblolly pine. The cDNA was amplified by PCR then ligated in pGEM T vector and transformed into *E.coli* bacterials. Bioinformatics tools were applied to analyze the specific cDNA sequences and their encoded protein sequences for their conserved functional domains, which may suggest those enzymes' possible functions. Sequences alignment was also carried out to predict their evolutionary positions and their possible functions by relation to their homologous genes in other organisms. In addition, these two genes' expression patterns in zygotic and somatic embryogenesis process were examined by RT-PCR. Finally, the two CERK, cDNAs were expressed in *E. coli* and their encoded proteins were purified. Enzyme assays were carried out for the CERK proteins and results were analyzed by TLC. Subsequent chapters showed the detailed information about this study:

Chapter 2 summarizes the background and fundamentals for this study. It includes the characteristics of Loblloly pine and the detailed process of embryo development of Loblloly

pine. It also summarizes the similarities and difference between zygotic embryogenesis and somatic embryogenesis in plant. The biochemical components of sphingolipids and their chemical structure, and their known functions in mammals and plants are reviewed in this chapter.

Chapter 3 outlines the objectives of the dissertation research.

Chapter 4 reports research results on serine palmitoyltransferase of Loblolly pine. Two full length serine palmitoyltransferases-like cDNAs were cloned; nucleotide and encoded protein sequences and their expression pattern were analyzed. Western blotting was also conducted. Domain analysis and western blotting results showed that the serine palmitoyltransferase subunit 2-like sequence might be the serine palmitoyltransferase 2 in Loblolly pine.

Chapter 5 demonstrates research results for Loblolly pine ceramide kinase (CERK). Two versions of CERK were cloned and a third version was also found. In addition to the analysis of nucleotides and the predicted protein sequences, the expression pattern in embryogenesis and their coding protein functions were also elucidated.

Chapter 6 summarizes the overall conclusions of this study and lists some recommendations for future research.

APPENDIX 1 presents the alignment of overexpressed Pt-CERK proteins.

APPENDIX 2 describes the main materials and methods used in this study in detail.

APPENDIX 3 describes the Fujifilm luminescent image analyzer LAS-1000 plus system.

APPENDIX 4 summarizes primers used in this study.

APPENDIX 5 lists alternative splicing results for SPT1 in Human, Arabidopsis, and Drosophila.

CHAPTER 2: Literature review

2.1. Loblolly pine is an important economic tree species in the southern United States

The plant species used in this study was Loblolly pine (*Pinus taeda L.*). Loblolly pine can grow as high as 30-35 m, with a diameter of 0.4-1.5 m and is a fast-growing southern pine species (Farjon 2001). Special specimens can reach 45 m tall (Farjon 2001). Loblolly pine can grow well in low wet places; but these trees are not limited to that specific habitat. With its ability to resist fire, Loblolly pines can also grow very well in the deep south to Florida (Farjon 2001).

2.1.1. Pine Embryogenesis is critical in supplying wood and biomass for sustainable energy

Although Loblolly pine grows generally in the southern United States, it is distributed widely, from the east to southern New Jersey, north to Tennesse, and west to central Texas as shown in Figure 2.1 (Baker & Langdon 1990; Farjon 2001). Isolated populations are also found in the areas with acidic sandy soil, which is surrounded by alkaline clays (Farjon 2001).

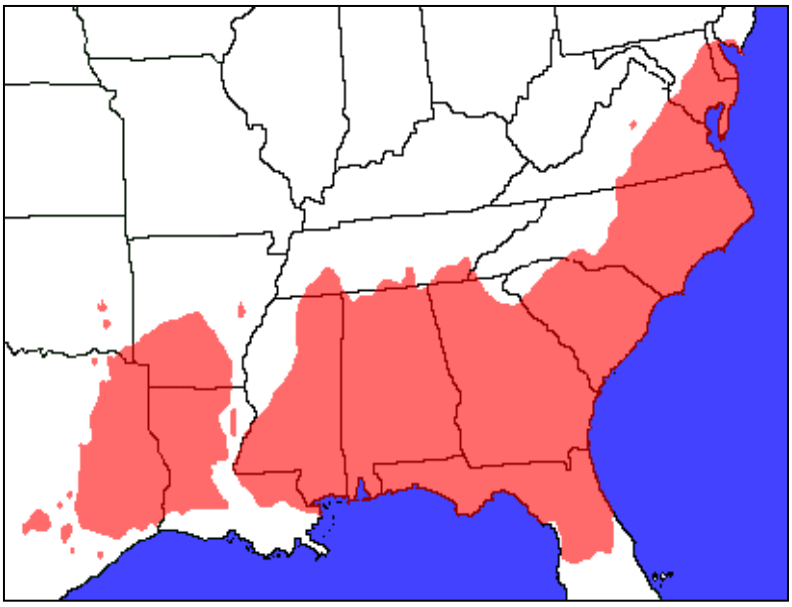


Figure 2.1. The natural geographic range for Loblolly pine (http://forestry. About.com/library/tree/bllobp.hit); Shaded region demonstrates the distribution range of Loblolly pine (Cairney & Pullman 2007)

Loblolly pine is a gymnosperm, which were the earliest seed plants (Cairney & Pullman 2007). Due to its fast growth in a wide variety of environmental conditions, the average rotation age of Loblolly pine is about 25 years (Schultz 1999). Commercially, this tree is planted in large plantations, accompanied by Slash pine (Farjon 2001). Because Loblolly pine has been one of the important tree species in southern east of the United State in providing lumber and biomass resource for pulp and bio-energy industries, the tree has been the focus of breeding programs which have resulted in great improvements in productivity.

Somatic embryogenesis (SE) has been known as an important technology with the potential for supplying tree plantation with a large numbers of genetically improved tree species for timber, fiber, and biomass. SE technology is making an increasing

contribution to the billions of Loblolly pine seedlings planted in the U.S. every year (Schultz 1999). However, some factors such as low somatic embryo maturation rate, and abnormal embryo formation limit the application of this technology in clonal propagation (Pullman et al. 2004). Obviously, a complete understanding of pine embryogenesis at the molecular and genetic level is critical to overcoming the above barriers.

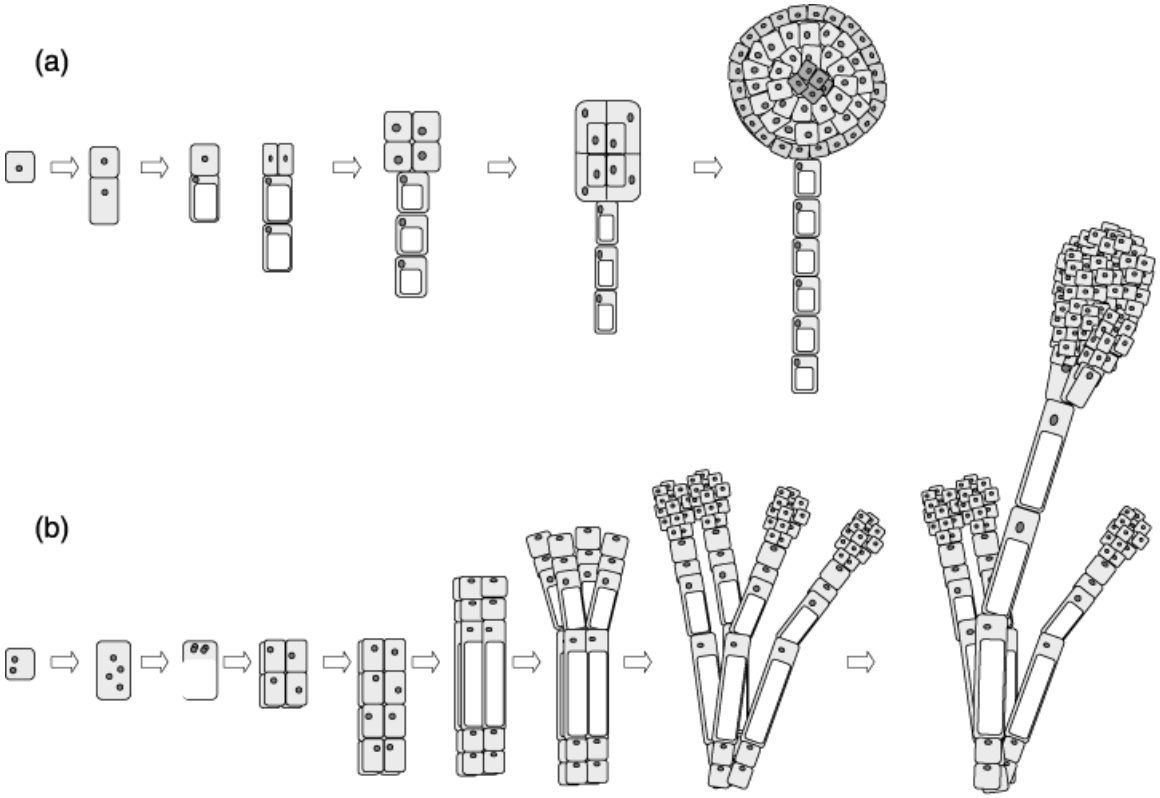


Figure 2.2. Diagrammatic representation of early events in embryogenesis in (a) *Arabidopsis thaliana* and (b) *Pinus taeda*
Dark circles: nuclei; shaded portion of cells: cytoplasm; unshaded portion of cells: vacuole. See text for details. Adapted from Cairney & Pullman (Cairney & Pullman).

2.1.2. Pine zygotic embryogenesis is different from embryogenesis in flowering plants

Zygotic embryogenesis is the first phase of differentiation and development in the plant life cycle (Cairney & Pullman 2007). Generally, this process consists of two phases: a morphogenic phase, which starts with gamete fusion to form the zygote followed by the

formation of embryo body; and a maturation phase, in which nutrient accumulation occurs to prepare the embryo for continuous desiccation and dormancy and to provide the food needed for early growth during germination (Figure 2.2 B) (Dogra 1967; Nagmani et al. 1995). The general process is the same for both gymnosperms and angiosperms (Figure 2.2 A), which are the two broad groups in plant kingdom classified according to the physical characteristics of embryogenesis. However, differences also obviously exist in embryogenesis between gymnosperm and angiosperm (Cairney & Pullman 2007). For example, the seeds of angiosperms develop inside the ovary of the mother tree. A double fertilization process occurs in angiosperm embryogenesis. This process begins with one haploid sperm cell fused to a haploid egg cell to generate the zygote; another sperm cell fuses with the diploid central cell to produce a precursor cell of the triploid endosperm (Cairney & Pullman 2007). Seeds of gymnosperms develop outside of the ovary. Gymnosperm embryogenesis starts with a single fertilization event within the ovule creating a diploid embryo, which develops within the haploid female gametophyte (Dogra 1967; Nagmani et al. 1995). Gymnosperms develop through a free-nuclear phase in which nuclei divides several times before cell wall formation (Figure 2.2).Embryo continues to divide and produce a four-tiered, 16-celled pro-embryo (Ciavatta et al. 2001). Within the mycropylar tip of the seed, the four cells in the distal tier develop into the embryo proper, and the lower tier develops into the suspensor (Cairney et al. 1999; Ciavatta et al. 2001). Loblolly pine forms multiple pro-embryos in early cell division in a process called cleavage polyembryony; As a result, each embryo proper can be cleaved into four embryos, each having its own suspensor (Spurr et al. 1949). It has been reported that among ten genera in the family Pinaceae, only *Cedrus*, *Keteleeria*, *Pinus* and *Tsuga*

were found to show cleavage polyembryony (Dogra 1967). Although poly-embryos form in the early development, only one of the embryos dominates and the remaining degenerate via program cell death in zygotic embryogenesis (Spurr 1949). During the embryogenesis of pine, the suspensor is identified as the first differentiated organ, which usually forms earlier than the embryo proper and survives till the cotyledonary stage in pine before its programmed cell death (PCD) (Yeung & Meinke 1993; Cairney & Pullman 2007). The suspensor has several functions: supporting and anchoring the embryo inside the megagametophyte; producing growth hormones such as gibberellins, and playing a role in transferring nutrients from female megagametophyte into the embryo (Yeung & Meinke 1993; Schmidt et al. 1997). Suspensors have been proven to have embryogenic potential; if the embryo proper is damaged, the growth of the suspensors will be stimulated and new embryos will form in these suspensors (Yeung & Meinke 1993). In contrast to angiosperms, gymnosperms can develop a large suspensor structure during somatic embryogenesis (Ciavatta et al. 2001). However, in Loblolly pine embryogenesis, it is most likely for the suspensors to start their program death before a mature embryo has formed (Figure 2.3). Unlike zygotic embryogenesis, programmed cell death and cell growth does not seem to be well controlled in somatic embryogenesis. Therefore, based on the research of model angiosperm, *Arabidopsis thaliana*, an investigation into the genetic events behind the embryogenesis of Loblolly pine might help identify the barriers in somatic embryogenesis of Loblolly pine.

2.1.3. A similarity exist in embryogenesis between gymnosperm and flowering plants

As mentioned previously, there are some differences between the processes of embryogenesis in gymnosperms compared with the embryogenesis process in

angiosperms (Cairney et al. 2006). To assess similarities or difference between angiosperm and gymnosperm embryo development, Cairney et al (Cairney et al. 2006) examined an Expressed Sequence Tags (EST) collection in pine for putative homologs of angiosperm genes involved in embryogenesis. It was found that out of 108 angiosperm embryogenesis-related genes, 83 homologs of these genes were present in pine, suggesting that pine and Arabidopsis have similar genes for embryogenesis (Cairney et al. 2006). Similar sequences for pine embryo development were also found in some other plants (Cairney et al. 2006). Further research by Cairney et al concluded that Loblolly pine had orthologs with strong sequence similarity to many Arabidopsis genes, which were involved in suspensor formation and early embryo development (Cairney & Pullman 2007). These results further confirm that both difference and similarity exist in embryogenesis between gymnosperms and angiosperms, an knowledge about the embryogenesis process in Arabidopsis is still a good reference for pine embryogenesis research.

A study by Mackay et al indicated that both mother-tree and father-tree in Loblolly pine embryogenesis had critical influence on the frequency of embryo initiation in somatic embryogenesis (MacKay et al. 2006). Maternal effects on embryogenesis were also reported in Arabidopsis (Vielle-Calzada et al. 2000). The importance of the suspensor formation has been demonstrated in the YODA (*yda*) mutant in later embryo development in Arabidopsis. A mutation *yda* gene, which encodes a Mitogen Activated Protein Kinase Kinase Kinase (MAPKKK), affects the zygote elongating. As a result, its first division is symmetric and leads to daughter cells in equal size. The identity of both

daughter cells causes the basal cell to lose its capacity to form suspensor, and instead, the cell becomes part of the embryo proper (Lukowitz et al. 2004).

2.1.4. Conifer somatic embryogenesis resembles zygotic embryogenesis but development stops prematurely or results in abnormal embryos

Somatic embryogenesis technology was first reported using carrot somatic tissue as the donor tissue for propagation (Steward 1958). Currently, somatic embryos can be initiated from many conifer species with various donor tissues (Gupta & Durzan 1986; Becwar & Pullman 1995). However, the most successful somatic embryogenesis donor tissue is reported to be immature zygotic embryos (Becwar & Pullman 1995). As described previously, gymnosperm embryogeny has a phenomenon called cleavage polyembryony: each embryo proper can be cleaved into a lot of individual embryos, each of which has its own intact suspensor (Becwar & Pullman 1995). Therefore, scientists can take advantage of this phenomenon to propagate embryos in appropriate media. Specifically, fertilized embryos from seeds are excised and placed on certain appropriate medium to allow the extrusion of embryogenic tissue from the micropylar end. Based on Pullman's research results (Pullman et al. 2004; Pullman et al. 2005; Pullman et al. 2006), many somatic embryos often form in the extruded tissue that can then be sub-cultured to a multiplication medium for maintenance, or to development medium and then maturation medium to guide the embryos to develop into a mature embryos (Figure 2.3). So this technology potentially provides many propagation advantages: 1) numerous equal genetic quality embryos will be produced in a less expensive manner; 2) liquid culture system can achieve a fast practical industry application; and 3) germplasm storage by cryopreservation can be utilized for a long term purpose. Thus, genetic gains of forest

trees can be captured through somatic embryogenesis. Great success has been achieved via somatic embryogenesis in producing high quality timber and fiber in the United States in the past. However, there are still some limitations for this (somatic embryogenesis) traditional method to produce large quantity of elite pine strain for plantation, although the somatic embryogenesis technology has attracted a lot of attention for forestry scientists to improve the production for decades. A few barriers, such as abnormal embryo formation and immature embryo producing still block the application of somatic embryogenesis in pine breeding (Pullman et al. 2004). Obviously, there is a need for additional genetic investigations of the embryogenesis process to understand and help solve these propagation problems in Loblolly pine.

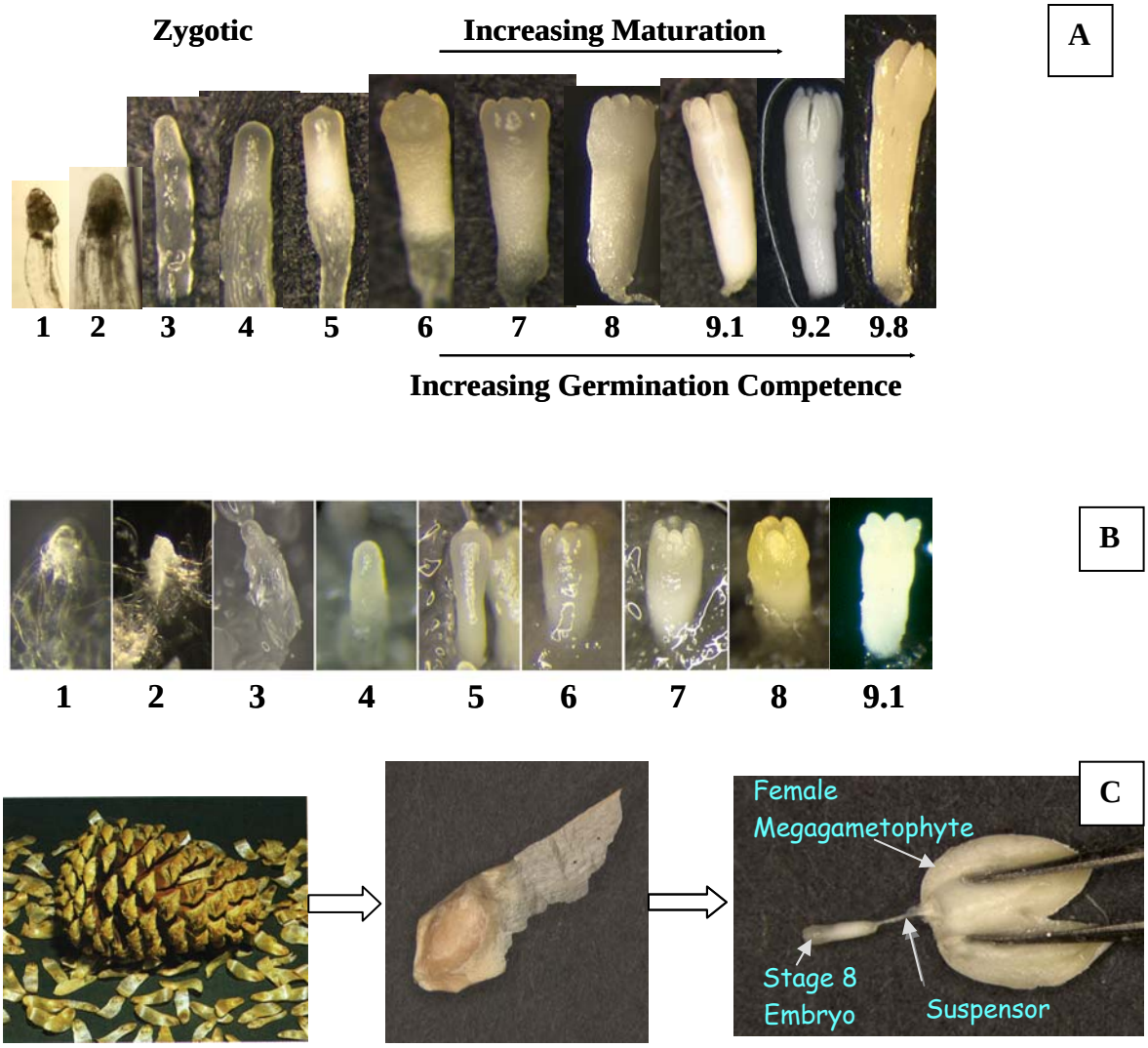


Figure 2.3. Stage s of Loblolly pine development (given by the number)
A) Sequence of Loblolly pine zygotic embryo development B) Sequence of Loblolly pine somatic embryo development showing similar developmental stages 1-9.1 C) The Loblolly pine cone, seed and decoated seed (Cairney & Pullman 2007)

2.2. Sphingolipids

Sphingolipids were first found to be components of brain in a treatise by Thudichum (Thudichum 1884) and a review of more recent finding about the discovered compounds from Thudichum's research (sphingomyelin, cerebroside, and cerebrosulfatide) can be found in Merrill (2002) (Merrill & Sandhoff 2001). Sphingolipids are essential

components of all animals, plants, fungi, some prokaryotic organisms, and viruses (Vesper & Schmelz 1999). All sphingolipids have a basic backbone structure, a sphingoid long-chain base (LCB) (Merrill & Sweeley 1996) like the one in Figure 2.4 and these have been studied in plants (Lynch & Dunn 2004). According to Merrill & Sweeley (Merrill & Sweeley 1996), more than 300 different sphingolipids have been identified structurally.

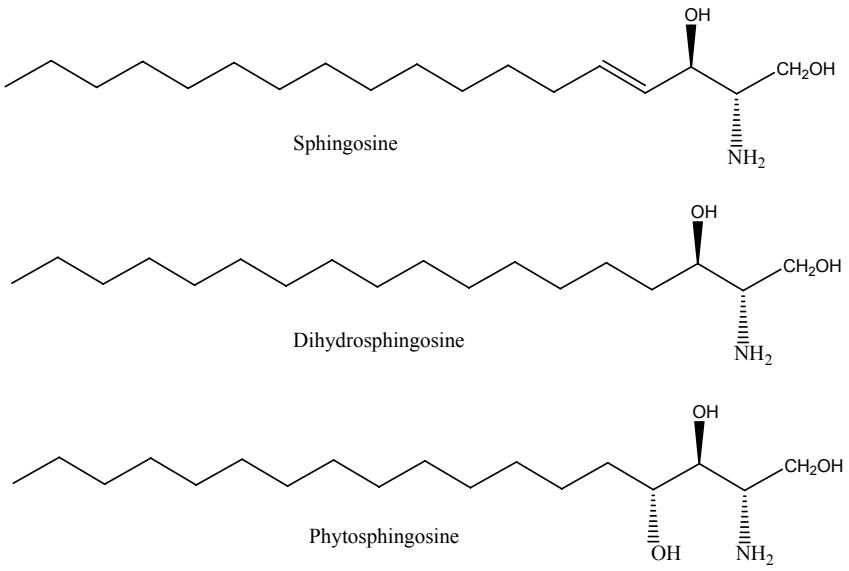


Figure 2.4. Three major types of natural sphingoid bases

Different organisms are characterized by different complex sphingolipids (Vesper et al. 1999). Generally there are three major types of sphingoid bases, sphingosine, dihydrosphingosine, and phytoshingosine as shown in Figure 2.4. Sphingosine is the main sphingoid base of sphingolipids in mammalian cells; the second most abundant sphingoid base in mammals is dihydrosphingosine (Hanada 2003). Phytosphingosine (4-hydroxydilhydrosphingosine) is the principal sphingoid base in plants and fungi (Hanada

2003). Sphingomyelins are the predominant sphingolipids in mammals; among them the neutral and acidic glycolipids are the dominant components (Hakomori 1983; Merrill & Sweeley 1996). The predominant complex sphingolipid of plant tissues is glucosylceramides and their cellular location is mainly in membranes and tonoplast (Lynch 1993; Lynch & Dunn 2004). In mammals, in addition to the membranes, sphingolipids are also major components of lipoproteins (Merrill et al. 2001). Sphingolipids have attracted researchers' attention in the past decades mainly due to three key discoveries. First, sphingolipids have been shown to be not only a membrane components but also the first and second messengers in a variety of signaling pathways (Merrill & Sandhoff 2002; Spiegel & Milstien 2003); secondly, they play critical roles in membrane microdomains, the 'lipid rafts' (Futerman 2004); and thirdly, they work as a marker of some environmental agonists (Hanada 2005).

2.2.1. Plant sphingolipids

Similar to the case in mammals, the complex sphingolipids in plants are created by the addition of various carbohydrate residues or phosphate-containing headgroups to a ceramide to form the nonpolar portion of sphingolipids. The abundant ceramide backbone in plants consists of a C18 long-chain base bound to a fatty acid by an amide linkage. The long-chain base and fatty acids varies in different numbers of hydroxyl groups and degree of unsaturation (Merrill et al. 2001; Merrill & Sandhoff 2002; Lynch & Dunn 2004). Typically, the fatty acid components contain 16-26 carbon atoms (Merrill & Sandhoff 2002; Lynch & Dunn 2004) in both mammals and plants. The dihydroxy sphinganine and the tryhydroxy 4-hydroxysphingaine, such as the phytoshingosine (d18:0) and

phytosphingosine (t18:0) are reported as minor constituents of complex sphingolipids, and also as free long-chain bases in plant tissues (Lynch & Dunn 2004). The configuration of double bond in sphingolipids exhibited various types: double bond in the Δ8 position could be *cis* or *trans*, whereas the double bond in the Δ4 position generally is the trans configuration. The abundant LCB sphingosine (trans-4-sphingenine) of mammals is found in a very low level in plant tissues (Chen et al. 2006). There are two principle complex sphingolipids, glucosylceramide (GlcCer) and inositiolphosphorylceramide (IPC) reported in higher plants (Lynch & Dunn 2004). The polar head group is in C-1 of the N-acyl long-chain base for both GlcCer and IPC (Figure 2.4) (Carter et al. 1961; Lynch & Dunn 2004).

2.2.2. Sphingolipid metabolism

Before discussing sphingolipid-mediated cell regulation, it is necessary to review the basic chemistry and biochemical pathways of sphingolipid metabolism. The pathway of de novo biosynthesis of sphingolipids in mammalian cells starts with the condensation of palmitoyl-CoA and L-serine to form 3-ketosphinganine in the endoplasmic reticulum and reactions continue in this compartment until the formation of ceramides by a series of reactions: reduction, acylation, and oxidative reactions (Figure 2.4) (Merrill 2002). Subsequent reactions occur in the Golgi apparatus, in which sphingomyelin (SM) and other complex sphingolipids such as glycolipids, gangliosides, and sulfatides are synthesized (Hannun et al. 2001; Merrill 2002). The principal enzyme in glycolipid synthesis is glucoceramide synthase (GCS) (Hannun et al. 2001), which is the precursor in the formation of many glycolipids and gangliosides (Merrill 2002). Sphingolipid metabolism is summarized in Figure 2.5 (Merrill 2002) .

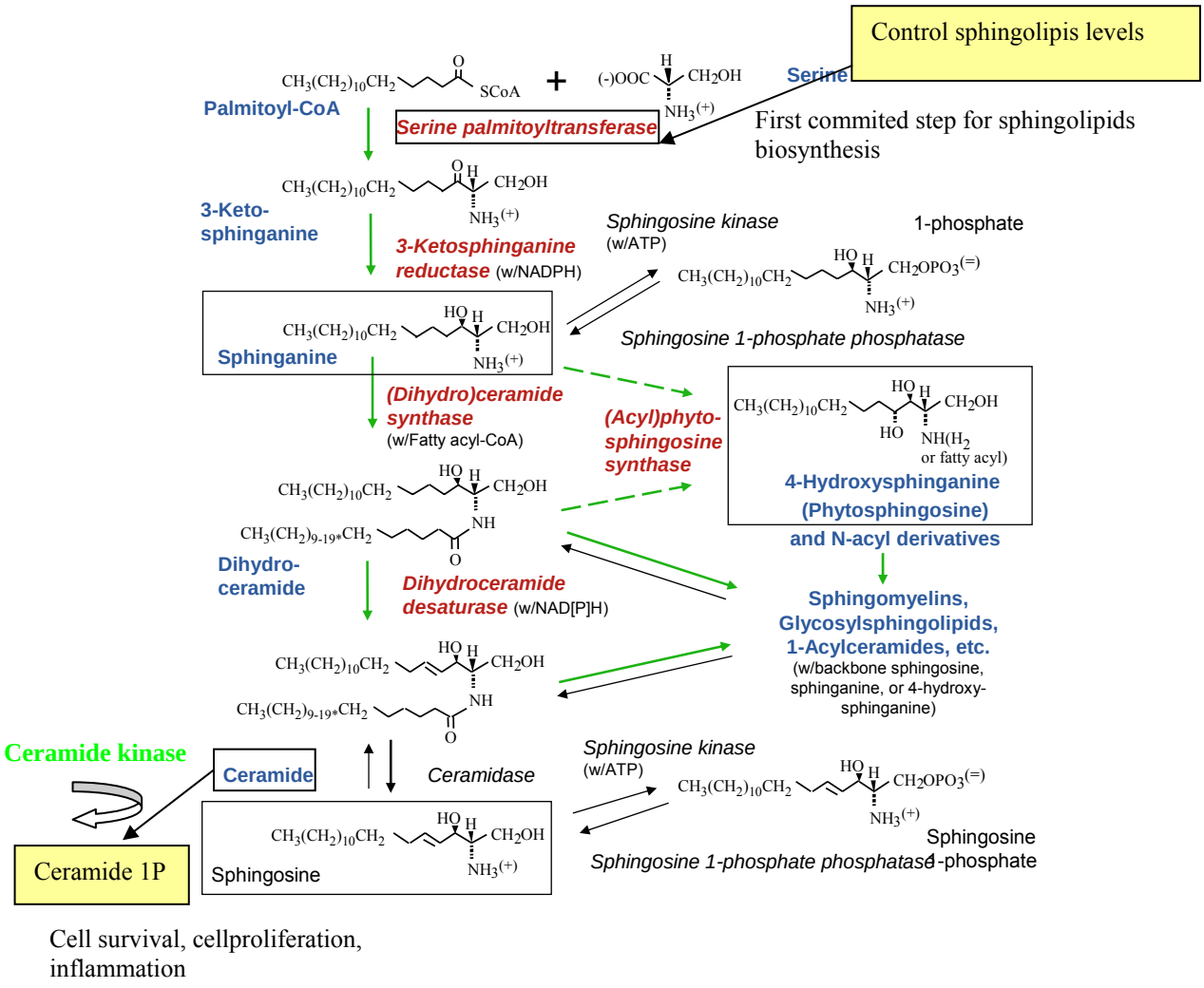


Figure 2.5. Sphingolipid metabolism pathway (Merrill 2002)

The sphingolipid metabolism pathway (Figure 2.5), describes both the main route for sphingolipid bio-synthesis and for the inter-conversion of the small active sphingolipids. For example, the breakdown of complex sphingolipids proceeds through degradation, hydrolysis and finally generates ceramide (Merrill 2002). Subsequently, ceramide can be deacylated to form sphingosine by ceramidase. On the other hand, sphingosine can be phosphorylated into sphingosine 1-phosphate (S1P) by sphingosine kinase or be salvaged to generate ceramide by ceramide synthase. The product, S1P can also enter the salvage pathway to be converted back into sphingosine through a

phosphatase. Alternatively, it may be degraded by a lyase to form a fatty aldehyde and ethanolamine phosphate, both of which may participate in glycerolipid pathways of metabolism (Hannun et al. 2001).

2.2.3. Sphingolipid metabolism in plants

Many enzymatic reactions in sphingolipids biosynthesis and degradation have been characterized *in vitro* using plant membrane preparations or lysates (Lynch 2000; Liang et al. 2003). Since almost all the sphingolipid enzymes are transmembrane or membrane attached, their enzymatic activity will often be lost during extraction with detergent. So far, no plant enzyme has been purified to homogeneity (Merrill 2002; Lynch & Dunn 2004). This barrier has greatly limited the progress in sphingolipid enzyme studies, especially in plants. Many sphingolipids biosynthesis reactions, which are involved in long-chain base and acyl-chain modification, have been not identified *in vitro*; some have been infered or characterized indirectly through protein labeling investigation (Kaya et al. 1984; Kojima 1993). However, the number of plant shingolipid genes, which have been cloned and functionally characterized in yeast and plant species is gradually increasing (Mitchell & Martin 1997; Tamura et al. 2001; Liang et al. 2003). BLAST search against the Pine Gene Index (http://compbio.dfci.harrard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=pine) for the homologs of the sphingolipid metabolism enzymes in Arabidopsis found that most enzymes in the sphingolipid metabolism pathway had homologs in Pine with very significantly smaller e-value. According to the analysis, it is likely that most sphingolipid enzymes are actively expressed and probably those enzymes and their regulated active sphingolipids function to some extent similarly in Loblolly pine

and their counterparts in mammals and yeast. Among all the metabolic enzymes, serine palmitoyltransferase and ceramide kinase (CERK) were the main focus in this study.

It is known that 3-ketosphinganine is formed through the condensation of palmitoly-CoA and serine catalyzed via serine palmitoyltransferase in the first committed step of sphingolipid biosynthesis (Snell et al. 1970). SPT is recognized as a key enzyme for regulating cellular sphingolipid level in mammalian tissues and is up-regulated under stress (Merrill & Jones 1990; Hanada 2003). Ceramide kinase, which phosphorylates ceramide to form ceramide -1 phosphate, is known as one of the conversion points in controlling ceramide level and in changing cell fate (mediating cell death and cell survival) (Obeid et al. 1993; Perry & Hannun 1998; Gijsbers et al. 1999).

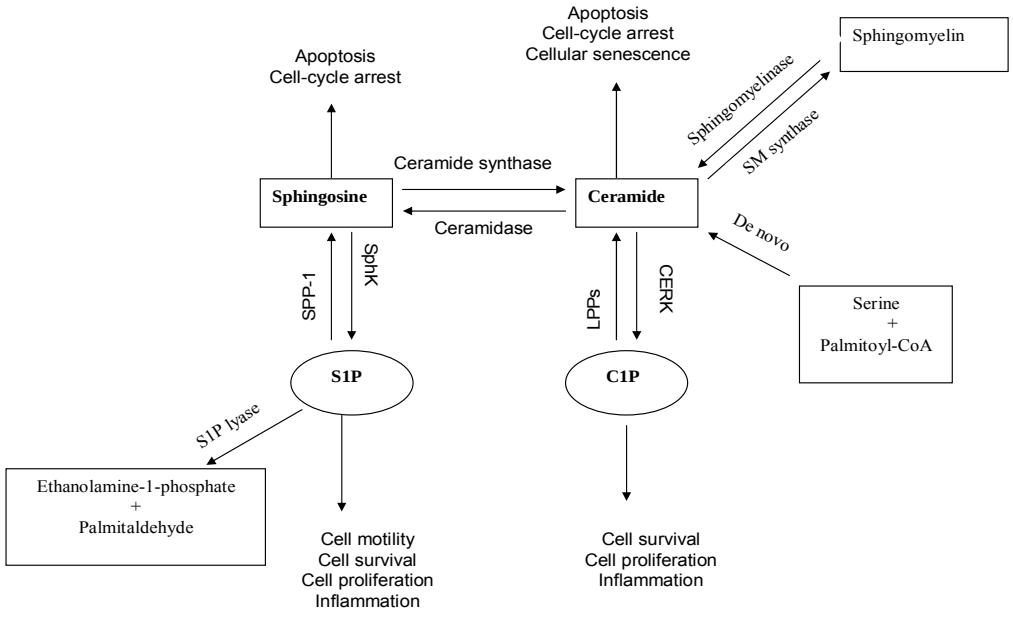


Figure 2.6. Bioactive sphingolipid metabolites

The schematic depicts the sphingolipid biosynthetic pathway. The interconvertibility of these molecules, the activity of a single enzyme in the depicted pathways may affect the fate of the cell (*Adopted from Charles E. Chalfant & Sarah Spiegel, 2005) (Chalfant & Spiegel 2005)*

2.2.4. Serine palmitoyltransferase

Serine palmitoyltransferase, a pyridoxyl-5'-phosphate dependent enzyme, is the first and rate-limiting enzyme in sphingolipids biosynthesis. It catalyzes the condensation of serine and palmitoyl-CoA to produce 3-ketodihydrosphingosine (3-KDS). 3-KDS is converted into ceramide through reduction of the keto by a reductase, acylation by dihydroceramide synthase, and the desaturation by the dihydroceramide desaturase. Subsequently, ceramide functions as a precursor for further conversation into complex sphingolipids such as sphingomyelins, glycosphingolipids, and some other biologically active small sphingolipids: sphingosine, sphingosine 1-phosphate, and ceramide 1-phosphate, which are shown in Figure 2.6. Some of these biologically active sphingolipids play vital roles in intracellular signal transduction and activation of cell death pathway and are implicated into cancer and other human disease. Therefore, as the key enzyme for regulating cellular sphingolipid levels, SPT attracts scientists' attention (Merrill & Sandhoff, 2002; Perry 2002).

SPT consists of two hetero subunits SPTLC1 and SPTLC2 in human (Hanada 2003), LCB1 and LCB2 in *Saccharomyces cerevisiae* (Buede 1991), and AtLCB1 and AtLCB2 in Arabidopsis (Tamura et al. 2001). SPT is believed to be located mainly in the endoplasmic reticulum (Yasuda et al. 2003) although a nuclear location has also been speculated (Batheja et al. 2003). In mammals, both SPT1 and SPT2 have a single highly hydrophobic domain in their N-terminal regions, which is named the transmembrane domain (TMD) (Weiss & Stoffel 1997). In yeast, three transmembrane domains have been reported (Han et al. 2004). The research conducted by Lynch (Lynch & Fairfield 1993) demonstrated that like its homolog SPT in human and Arabidopsis, SPT activity in

squash (*Cueurbita pepo L.)* pericarps exhibited substrate specificity for palmitoyl-CoA in a pyridoxal 5'-phosphate-dependent reaction, and the enzymatic reaction occurred in the endoplasmic reticulum (Tamura et al. 2001; Perry 2002). Therefore, the catalytic mechanism and sub_cellular localization of SPT may be conserved among various organisms including animals, fungi and plants (Tamura et al. 2001).

Much is not known about SPT even in humans. For example, one can find multiple transcripts for putative SPTs in the NCBI gene database; four putative SPT1 transcripts are present in humans. One of them Y08685, 2780bps in length and encoding 53 kD protein, is the catalytic subunit 1 in human. The other three are AAH68537 with 2742bps, NM_178324 with 998 bps and XP_001130723 with 195 bps; among them the first two transcripts (AAH68537 and NM_178324) have 96% and 32% identity to the 53 kD transcripts, respectively. The version of XP_001130723 is part of the 53kD transcripts. However, the functions of above three versions have not been reported in literature. Like human SPT1, four transcripts of human SPT2s have been found to exist. The predicted amino acid sequences for all four transcripts are different. Among them, two versions of human SPT2 (NP_018327 and Y08686) have been characterized and identified, indicating that both have the catalytic function, although their amino acid sequences are different (Hornemann et al. 2006).

The same problem exists for plants. Arabidopsis LCB2 protein contains a unique sequence: ^{307}GTFTKSFG^{314}, which is related to the pyridoxal phosphate-binding motif that is commonly found in members of the α-oxoamine synthase subfamily, such as 2-amino-3-ketobutyrate CoA ligase (Mukherjee & Dekker 1990) and 8-amino-7-oxononanoate synthase (Alexeev et al. 1998). The GTFTKSFG motif is completely

conserved among the predicted LCB2 proteins from *S. serevisiae*, Arabidopsis, and human, suggesting that these LCB2 polypeptides constitute catalytic subunits of SPT. So far, no reports show whether homology of SPT exists in pine.

2.2.5. Ceramide kinase, a key enzyme in mediating ceramide conversion to ceramide 1-phosphate plays vital roles in cell fate

Ceramide kinase is an ATP dependent kinase, which generates ceramide 1-phosphate (C1P), which is able to eliminate the cell death-inducing effects of ceramide in certain levels (Bajjalieh et al. 1989).

In animals, the activity of ceramide kinase was first identified by Bajjalieh and co-workers in brain synaptic vesicles (Bajjalieh et al. 1989) and then found in human leukemia (HL-60) cells (Bajjalieh 1989; Kolesnick 1990). Ceramide kinase mediates the level of ceramide and its activities are induced during pathogenesis (Gomez-Munoz 2004; Chalfant & Spiegel 2005). Its product, creamed 1-phosphate (C1P) was identified as a mediator of arachidonic acid release and prostanoid synthase and was proved to be a direct activator of cytosolic phospholipase A2 (Pettus et al. 2003) (Figure 2.7). Research by Mitsutake and Igarashi (Mitsutake & Igarashi 2005) demonstrated that ceramide kinase and ceramide-1-phosphate participated in the deregulation process in mast cells and proved that this process was calmodulin dependent (Mitsutake & Igarashi 2005). Furthermore, C1P was reported to possess both mitogenic and anti-apoptotic effects in mammals (Bajjalieh et al. 1989). Similar results were obtained by Gomez-Munozz that C1P blocks DNA fragmentation as well as caspase activation (Gomez-Munoz 2004). C1P shares structural homology with phosphatidic acid, which has been shown to be highly

fusogenic (Blackwood et al. 1996). So CERK might also be involved in the cellular fusion.

In summary, both ceramide-1-phosphate and ceramide kinase have been involved in the regulation of vital cellular processes, such as cell proliferation (Gomez-Munoz et al. 1995), apoptosis (Gomez-Munoz 2004), phagocytosis (Hinkovska-Galcheva et al. 1998), inflammation (Pettus et al. 2003), and membrane fusogenic activities (Blackwood et al. 1996).

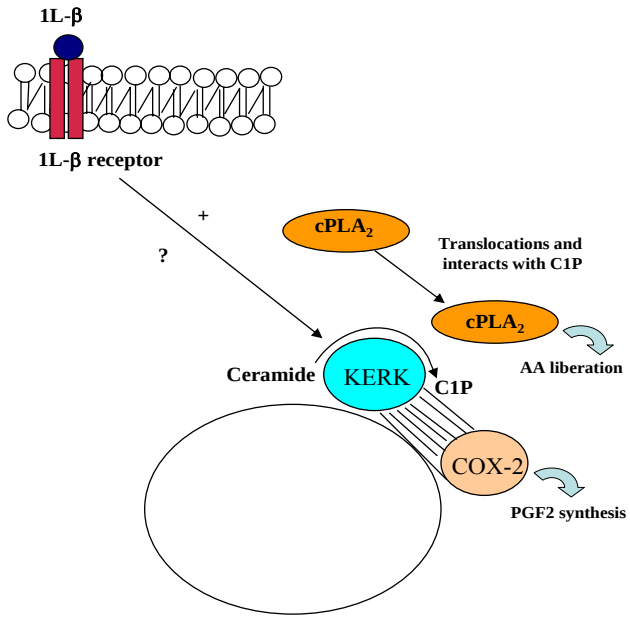


Figure 2.7. Roles of CERK and C1P in eicosanoid synhesis
The prostanoid biosynthesis in response to IL-2β, involves the activation of cPLA2 and generation of arachidonic acid (AA), which requires CERK and the generation of C1P (Adapted from Chalfant & Spiegel 2005)

Recently, a ceramide kinase (*h*CERK) from humans was characterized and demonstrated to phosphorylate a number of ceramide substrates with much higher activity than sphingosine substrates (Sugiura et al. 2002). The *h*CERK encodes a protein of 537 amino acids that has a catalytic region with a high degree of similarity to the diacylglycerol kinase catalytic domain. *h*CERK possesses a pleckstrin homology (PH) domain, which is thought to have several different functions and to participate in the regulation of the proteins that possess this domain (Gibson et al. 1994). For example, PH domain-containing proteins can bind to the β/γ subunit of heterotrimeric G-protein (Toubara et al. 1994), to phosphatidylinositol-4, 5-bisphosphate, and to phosphorylated tyrosine residues (Toubara et al. 1994). So, the PH domain of CERK might be important for membrane anchoring and regulate its catalytic activity (Sugiura et al. 2002).

Interestingly, some reports about the subcellular distribution of CERK are contradictory (Bajjalieh et al. 1989; Sugiura et al. 2002), for example, that CERK activity is located in the cytosol plasma membrane (Mitsutake 2005) or the Golgi (Carre et al. 2004). CERKs are widely present in diverse organisms from plants, nematodes, insects, to vertebrates.

One of the functions that have been proposed for CERK is the control of programmed cell death (Liang et al. 2003). The processes of programmed cell death in plant have been identified to play critical roles in development and disease resistance (Filonova et al. 2000; Koonin & Aravind 2002; Bozhkov et al. 2005). The polyembryony in gymnosperm is a process that multiple embryo initially forms and eventual only one survives and become dominant (Filonova et al. 2000). It has been found that the elimination of subordinate embryos is carried out via PCD and occurs in a wave spreading pattern from

the embryo base to tip (Filonova et al. 2000). In embryogenesis process, the suspensor structure is degraded by PCD when embryos develop to the cotyledonary stage in conifers (Cairney & Pullman 2007). Programmed cell death also occurs in megagametophytes during embryogenesis of Norway spruce, because with embryo growth and enlargement, room is needed for the embryo to grow deeper into the megagametophyte (Filonova et al. 2002; Cairney & Pullman 2007). It is thus possible that ceramide kinase functions in a similar way to its orthologs in animals i.e. as a cell survival mediator in balancing normal or injury-induced programmed cell death during embryogenesis of Loblolly pine. The Loblolly pine ceramide kinase may also mediate suspensor's function in embryo development. If so, it will be a useful focus point in determining the difference between zygotic embryogenesis and somatic embryogenesis in Loblolly pine.

In plants, a mutant Arabidopsis, which was selected for its increased sensitivities to pathogen attack, had been proven to be defective in a gene encoding ceramide kinase. This CERK mutant Arabidopsis (ADC5) plants develop normally at the beginning, but display obvious cell death compared with the recombinant plants with bacterial pathogen *Psudomonas syringae* attack (Liang et al. 2003). Furthermore, the later cell death of this mutant plant is largely dependent on external stress and defense signaling, which is controlled by the plant hormone ethylene and the phenolic salicylic acid, respectively (van Doorn & Woltering 2005). CERK functions as a conversion point between cell death (ceramide) and cell survival (C1P) (Liang et al. 2003, Gomez-Munoz et al. 1995). In plant pathogenesis, it is also known that the balance between ceramides and their phosphorylated derivatives is important in modulating cell survival and cell death, which

is controlled by the defense and stress signals salicylic acid and ethylene (Chalfant & Spiegel 2005). Another report (Greelman & Mullet 1997) demonstrated that wounded tomato leaves produced systemin to stimulate biosynthesis of jasmonic acid, which is a plant growth regulator with diverse effects. The research results reported by Clausen et al are inconsistent with Greelam's finding that wounded tomato plants activate corresponding genes to produce ethylene and as a result generate systemin and turn on the biosynthetic pathway for jasmonic acid (Clausen et al. 1996). During Loblolly pine somatic embryogenesis, the abnormal growth conditions of tissue culture might cause Loblolly pine to produce plant hormones, such as, ethylene, jasmonic acid to speed up suspensor cell programmed death before embryos completely mature. Abnormal somatic embryogenesis development might, therefore, be a result of an imbalance between ceramide and C1P due to abnormal ceramide kinase activity.

2.2.6. Sphingolipids are important players in embryo development and organ formation

During embryogenesis in mammals, it has been demonstrated (Bird & Kimber 1984; Kudo et al. 2004) that some sphingolipids in the membrane do play important roles in cell-cell interaction and recognition. Stage-specific embryonic antigen 1 (SSEA-1) is a complex sphingolipid that is widely expressed on the surfaces of mammalian cells and considered to be involved in cell-cell interactions during embryogenesis, differentiation, and the neuron-developmental process (Merrill & Sandhoff 2002). Research by Hojjati et al indicated that serine palmitoyltransferase activity was essential for embryonic development because homozygous SPT1 and SPT2 knock-out mice die during embryogenesis (Hojjati et al. 2005). Mutant flies with SPT partially deficiency develops into adults with abnormalities in various external organs; however, such abnormalities

can be rescued by feeding flies with sphingosine (Adachi-Yanada et al. 1999). Research by Isabel et al (Isabel et al. 2004) also suggestes that ceramide kinase are paralleled by the anti-activation of pro-apoptotic pathways related to embryogenesis. Those genetic findings suggested that sphingolipids are essential not only for growth of cells but also for the development of animals (Hanada 2003). So far, there is no literature report about the effect of sphingolipids in embryogenesis in pines. BLAST searching against the Pine Gene Index for the homologues of the sphingolipid metabolic enzymes in Arabidopsis, reveals that most enzymes in the sphingolipid metabolism pathway have homologs in Pine with significantly low E-value (lower than $8.6e^{-20}$). Therefore, I have chosen to investigate the key enzymes, serine palmitoyltransferase and ceramide kinase from embryos of Loblolly pine; specifically to isolate, to clone, and to characterize their functions during embryogenesis of Loblolly pine.

2.3. Significance

As previously described, Loblolly pine is an important commercial conifer species in the United States for the supply of wood and biomass for paper industry and sustainable energy. However, traditional breeding methods have shown limitation in production of large quantities of elite pine strains for plantation forestry. Although great success has been achieved via somatic embryogenesis in producing high quality timber and fiber in the United States, abnormal embryo formation and immature embryo production still restrict the application of somatic embryogenesis in pine breeding (Pullman 2004) . Obviously there is a need for the genetic investigation in embryogenesis process to understand the main reasons and help solve these propagation problems in Loblolly pine. So it is necessary to integrate the tissue culture into

molecular biology research when investigating embryogenesis in Loblolly pine, especially the molecular and cell events behind the embryogenesis. Investigation into the roles of sphingolipids in embryogenesis of Loblolly pine is a part of these research works and will provide new insight into plant embryo development.

CHAPTER 3: Research objectives

3.1. Specific objectives

Although there are some reports about sphingolipid gene in Arabidopsis, knowledge about sphingolipids in gymnosperm is still limited. A BLAST search against the pine cDNA library for the homologs of the sphingolipid metabolism enzymes in Arabidopsis (Table 3.1) found that almost all enzymes in the sphingolipid metabolism pathway have homologs in Pine with very significantly low E-value (lower than $8.6e^{-20}$). Therefore, it is hypothesized that the metabolism enzymes exist in embryogenesis of Loblolly pine and might play some of the same roles as their homologs in Arabidopsis and mammals.

The overall objectives of this study were to investigate serine palmitoyltransferase and ceramide kinase and their functions in embryogenesis of Loblolly pine. The specific research objectives were shown as follows:

Sub-objective 1: Clone the full length cDNAs of ceramide kinase and serine palmitoyltransferase; Compare and contrast the structural differences of these enzymes with their orthologs in other organisms

In these sub-objectives, full length cDNA transcripts of genes that encode putative serine palmitoyltransferase and ceramide kinase have been cloned using RNA isolation, RT-PCR, and cDNAs cloning techniques.

Table 3.1. Multiple SPT transcripts in various organisms BLAST E-value compare sequences of pine transcript assemblies with Arabidopsis reference RNA
ND. Not determined. (Adapted from Dunn et al. 2004)

Enzyme	Pine homologs	Yeast code	MIPS code	E Value	Functional characterization status
Serine palmitoyltransferase	TC45727 TC44537	LCB1 LCB2	At4g36480 At3g48780 At54g23670	1.10E-129 1.30E-118	Chimeras of the yeast and Arabidopsis *lcb2* genes complement the *lcb2* Δ null mutant
3-Ketodihydrosphingosine reductase	TC40440 TC40441	TSC10	At3g06060 At5g19200	1.80E-86 2.90E-86 2.90E-86	ND
Sphinganine C4 hydroxylase	TC49647	SUR2	At1g14290 At1g69640 At4g04930	1.60E-40	Arabidopsis At1g14290 complements the yeast *sur2* Δ Mutant (Sperling et al. 2000)
Fatty acyl-CoA carrier	TC41389 TC41391	ACB1	At1g31812	1.10E-24 1.60E-22	ND
Ceramide synthase	TC44043 CF394959	LAG1 LAC1	At3g19260 At1g1358	1.80E-38 3.10E-35	ND
Components of elongase comple required for VLCFA synthesis	TC43660 TC56392 TC49054	ELO1 ELO2/FEN1 ELO3/SUR4	At3g06460 At3g06470 At1g75000	3.30E-52 6.90E-33 5.90E-53	ND
Probable condensing Enzymes (3-keto-acyl-synthases)	TC56392 TC49054		At4g36830	3.30E-56	
Component of elongase complex required for VLCFA synthesis 3-Keto reductase	TC53725	YBR159w	At1g67730 At1g24470	2.50E-67 9.20E-70	Heterologous expression of At1g67730 in yeast complements the *ybr 159* Δ mutant (Han et al. 2002)
Component of elongase complex required for VLCFA synthesis. Trans 2,3-enoyl reductase	TC42175	TSC13	At3g55360	9.90E-131	Heterologous expression of At3g55360 in yeast complements the *tsc* l3-1 mutant (Gable et al. 2004)
VLCFA α –Hydroxylase	TC47121 TC47121	SCS7/FAH1	At2g34770 At4g20870	7.20E-90 5.30E-78	At2g34770 complements the *scs*7 Δ yeast mutant (Mitchell and Martin, 1997)
LCB-1-phoshate lyase	TC54565	DPL1	At1g27980		ND
LCB-1-phosphate-phosphatases	TC50375	LCB3	At3g58490	8.60E-20	ND
LCB kinases	TC53982	LCB4 LCB5	At4g21540 At5g23450	4.00E-104	At5g23450 (AtLCBK1) was shown to phosphorylate sphinganine in *E coli* extracts (Nishiura et al. 2000)
Ceramidases	TC53264	YDC1	At4g22330	2.00E-102	ND
Accelerated-cell-death 5	TC45250	ACD5	AY362552 At5g51290	1.2E-38	
Accelerated-cell-death 11	TC44539	Not found	Atag34690	2.00E-65	Acd 11/jar1-1;acd11/ein2-1 (Arabidopsis)
Prototypical Galpha	TC43490	GAP1	At2g26300 GAP1	4.60E-85	Not found
Note: All sphingolipid metabolism enzymes have homologs in Loblolly pine. All Arabidopsis genes are named by their MIPS code number, in which the first digit indicates the chromosome where the gene is present, and the continue five-digit number is the unique identifier for each ORF. The Blast e-value is between Pine and Arabidopsis. ND. Not determined (Adapted from Dunn et al. 2004)					

Sub-objective 2: Analyze the evolutionary position of these two enzymes and do alignment to compare and contrast these enzymes in Loblolly pine with their orthologs in *Arabidopsis theliana*, yeast, and mammals

In these sub-aims, the amino acid sequences of open reading frames of these putative enzymes and their counterparts in other organisms have been first aligned using ClustalW with Biology WorkBench 3.2.(http://workbench.sdsc.edu). The alignments have been then visually refined. Only the regions of ORF alignments were used in the subsequent phylogenetic analysis as described in the experiment design sections.

Sub-objective 3: Analyze the functions of recombinant CERK genes to determine whether the enzymes function similarly to their counterparts in Arabidopsis or even same as their counterparts in mammals.

To realize this aim, CERK proteins were overexpressed and purified by employing pET28a^{+} vectors, which have his6-tags, with target CERK ORFs and transforming the recombinant plasmids into *E. coli* strain BL21 for large-scale expression. Protein purification followed the procedures of 5 mL TALON® Single Step Column Purification. Then, enzyme assays were conducted for above purified proteins based on literature reported methods (Bajjalieh and Batchelor 1999). Enzyme reaction products were analyzed by TLC.

Sub-objective 4: Characterize and quantify the expression amounts of the serine palmitoyltransferase and CERK mRNAs during embryo development and determine whether the different transcripts within each enzyme are developmentally specific. Establish an expression profile for CERK mRNAs

In this sub-aim, gene specific primers were designed and used for PCR to amplify ORFs or their specific domains within protein coding region to quantity their expression patterns at different stages during embryo development of Loblolly pine.

CHAPTER 4: Identification of putative serine palmitoyltransferase transcripts in Loblolly pine and changes in embryogenesis

Abstract

Sphingolipids are important as cell membrane components and function as critical signaling molecules in animals. Their role in angiosperm development is being elucidated but no data is available for evolutionary ancient gymnosperms. By surveying databases this study demonstrates the existence in loblolly pine of candidate genes for sphingolipid biosynthesis. Putative (Pt-) cDNA clones for loblolly pine SPT1 and SPT2 were isolated and analyzed. The longest version of Pt-SPT1 (2,223 bps) encodes a protein with 484 amino acids which is 81% similar to Arabidopsis SPT1, while Pt-SPT2 (2,396 bps), encodes a protein of 493 a.a. which has 88% similarity to SPT2 in Arabidopsis**.** Both predicted pine proteins contain strongly conserved serine palmitoyltransferase function domains. We also cloned a few smaller versions of mRNAs of serine palmitoyltransferase subunits 1 in Loblolly pine (Pt-SPT1) two of which (822 bps, 756 bps) encode the same 90 a.a. protein. The small protein with 90 a.a. lacks many serine palmitoyltransferase function domains but contains a sterile alpha motif (SAM) domain. The smaller transcripts may be produced from the Pt-SPT1 gene by alternative splicing. The small protein may act as a dominant negative form of SPT1 and/or may have independent functions. Subsequent examination of databases revealed additional smaller SPT1-related mRNAs in yeast, Arabidopsis, Drosophila and Human. This raises the possibility of additional forms of SPT1 participating in regulating sphingolipid biosynthesis. Although the SPT1 and SPT2 subunits are thought to function in a 1:1 ratio,

the mRNA levels of SPT1 and SPT2 do not parallel one another which implies a post transcriptional regulation of SPT subunit production.

4.1. Introduction

Sphingolipids are important components of cell membrane and function as critical signal molecules in animals for cell growth, cell death, embryogenesis and neuronal development (Chalfant & Speigel 2005, Herr et al. 2003, Kudo et al. 2004, Marasas et al. 2004, Tani et al. 2007). The importance of these metabolites is indicated by a range of human diseases which can be ascribed to defects in sphingolipid metabolism (Kolter & Sandhoff K 2006, Kacher & Futerman 2006). For example, loss of serine palmitoyltransferase (SPT) activity in invertebrate's results in embryo abortion (Adachi-Yamada et al. 1999), Ceramide, the precursor of all sphingolipids, can cause cell cycle arrest and activate cell apoptotic pathway and its level in mammals is regulated by SPT (Obeid et al. 1993, Perry & Hannun 1998)

The influence of sphingolipids upon plant growth and development has been understudied but is now an area of active research (Liang et al. 2003, Dunn et al. 2004, Brodersen et al. 2005, Chen et al. 2006). This work, however, has been confined to angiosperms (flowering plants) and little is known about evolutionary ancient plants such as Gymnosperms. Loblolly pine (*Pinus taeda* L.) is a species of considerable ecological and economic importance. The cell and molecular biology of embryogenesis in conifers, such as loblolly pine, exhibit many features which distinguish pine embryo development from the more widely studied processes in angiosperms (Cairney & Pullman 2007). Multiplication of embryos in culture via Somatic Embryogenesis (SE) has permitted physiological and molecular analysis of embryo development. SE is viewed as a viable

means of propagating superior genotypes for reforestation and biomass provision (Schultz 1999). The study of embryogenesis in conifers thus has both fundamental and practical value. The production of plantlets from tissue culture, however, is very genotype dependent and misshapen and aborting embryos are common (Schultz 1999). Determining signals that direct embryogenesis in vivo and correlating those with specific proteins come to age for Loblolly pine embryogenesis.

4.2. Materials and methods

4.2.1. Materials

Zygotic and somatic embryos

Pine cones were harvested weekly from open-pollinated loblolly pine (*Pinus taeda* L.) 7-56 mother trees; collections occurred from June 20th to September 15th in 2004 and 2005 in a commercial pine orchard in Lyons, Georgia, US. Megagametophytes were isolated from seeds, dissected and embryos were removed and immediately flash frozen in liquid N_2, then stored at -70 ^{o}C prior to use. The staging system to determine loblolly pine embryo development has been described previously (Pullman and Webb 1994, Ciavatta et al. 2001). Somatic embryo cell lines were initiated from immature megagametophytes isolated from loblolly pine seed. Cells were subcultured weekly. Maturation of somatic embryos was achieved using media and methods of Pullman et al (Pullman et al. 2003).

Antibodies raised against Arabidopsis SPT2 protein were a generous gift from Dr. Edgar B. Cahoon in USDA-Agricultural Research Service, Donald Danforth Plant Science Center, St. Louis, MO.

4.2.2. Molecular methods

RNA was isolated by using the RNeasy Plant Mini Kit (Quiagen, Valencia, CA). First strand cDNA was generated using the SMART RACE cDNA Amplification Kit (BD Clontech, Mountain View, CA),

The Primer 3 software program (http://frodo.wi.mit.edu/) was used to design gene specific primers for 5'-RACE and 3'-RACE reactions (BD Clontech, Mountain View, CA) based on EST sequences in the Pine Gene Index (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=pine) The 5' RACE Gene Specific Primer for SPT1 (5'-TGGGTCCCTGAGCCACTACATCCA-3') was derived from BE123605, 3' RACE Gene Specific Primer for SPT1 (5'-TGCTGGTATGGGGTATGCTTTGGCA-3') from DR024381; The 5'RACE Gene Specific Primer for SPT2 (5'-TTGCCGTTTTTCAGCACAACACACC-3') from DR688896, and the 3'RACE Gene Specific Primer for SPT2 (5'-TGCAGGAAAGGCAACTGTCACCACTGC-3') from CO200178.

Full-length cDNA clones were isolated as single molecules by RT-PCR using gene specific primers based on sequence from 5'-RACE and 3'-RACE clones. The forward and reverse primers were as follows: Pt-SPT1F (5'-TCCCGCAGCACACCCTTGACCA-3'); Pt-SPT1R (5'-TTGCCTGACCTAGCTGAACATGAATGA-3'), Pt-SPT2F (5'-CGAATGCGTGCAAGCTTTTGAGCTCCT-3') and Pt-SPT2R (5'-CGCCACATCAAAATGTGGTGCCATTC-3').

PCR products were cloned into the pGEM-T Easy Vector System (Promega, Madison, WI).

4.2.3. Microsomal membrane isolation

The fresh frozen embryos were ground into powder and immediately placed into 1 eq (w/v) of cold homogenizing medium containing 500 mM Sucrose, 50 mM Hepes-KOH (pH7.8), 5 mM EDTA, 2 mM DTT, 0.5% PVP, and 0.1% BSA. The tissue was homogenized using a Polytron PT 20, by applying five 15-second pulses. The homogenate was filtered through three layers of damp cheesecloth and centrifuged for 30 min at 13,000g. The resulted supernatant was centrifuged for 60 minutes at 105,000g. The microsomal pellet was re-suspended and homogenized in cold 10Mm Hepes-KOH (pH 7.8), 2 mM EDTA, and 4 mM DTT, and 20% 9w/v) glycerol using a Teflon/glass homogenizer. The protein concentration of the suspension was determined using method of Bradford (Bradford et al. 1976).

4.3. Results and Discussion

4.3.1. Loblolly pine contains gene necessary for sphingolipid metabolism

The Pine Gene Index DFCI Pine Gene Index (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=pine) was surveyed for ESTs which could encode enzymes for sphingolipid biosynthesis. BLAST searches were conducted using the sequences of Arabidopsis mRNAs annotated as sphingolipid metabolic enzymes. Loblolly pine ESTs with strong sequence similarity (E-value lower than 8.6 e^{-20}) were found for almost all genes examined (Table 3.1 in page 28). Thus genes for many of the enzymes that have been characterized in yeast and Arabidopsis appear to be present in the loblolly pine transcriptome.

Serine Plamitoyltransferase (SPT) is a key enzyme in sphingolipid biosynthesis catalyzing the decarboxylative condensation of L-serine and palmitoyl coenzyme A to form 3- ketodihydrosphingosine (Snell et al. 1970, Williams et al. 1984). SPT regulates the sphingolipid content of mammalian tissues and of cultured cell lines (Merrill & Jones 1990). Although sphingolipids are primarily present in eukaryotes, they are also found in some bacteria, mainly in *Sphingomonas* species. Studies in eukaryotes have demonstrated that SPT consists of two subunits encoded by the long chain base LCB1 and LCB2 genes (Buede et al. 1991, Tamura et al. 2001). The SPT enzymes functions as a heterodimer; LCB2 (SPT2) encodes the catalytic domain, while LCB1 (SPT1) stabilizes the complex and may determine substrate specificity (Hananda 2003). A recent study demonstrated that when co-expressed with *Arabidopsis* LCB2, a predicted gene for *Arabidopsis thaliana* LCB1 encoded the subunits of SPT rescued the sphingolipid long-chain base auxotrophy of *Saccharomyces cerevisiae* SPT mutants (Chen et al. 2006). Partial RNA interference (RNAi) suppression of AtLCB1 expression caused a significant reduction in plant size. This was primarily caused by reduced cell expansion but without reduction in weight in the RNAi suppression plants (Chen et al. 2006).

This study found several pine EST whose sequence resembled spliced transcripts potentially encoding alternative forms of SPT1, an intriguing observation given the importance of the enzyme subunit. A relation to the production of different forms of SPT1 mRNA to different phases of embryo development would be of interest; therefore complete cDNA copies of SPT1 mRNAs were obtained by using a combination of 5'- and 3'-RACE (BD Clontech, Mountain View, CA), cloning using EST sequences from public databases.

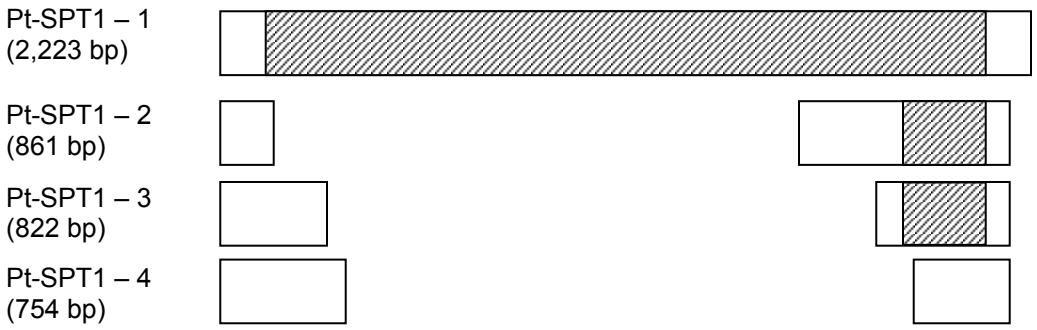


Figure 4.1. Diagram of Pt-SPT1 cDNA alignment
Shaded boxes: open reading frame; unshaded boxes: untranslated region. Sequences are identical in regions of overlap

Figure 4.1 shows nucleotide alignment of Four SPT1-like mRNAs isolated by RACE reactions. The structure of these mRNAs, which exhibit sequence identity in areas of overlap, suggests that they are probably produced by alternative splicing. Furthermore, when aligning those four transcripts with the chromosome4 (NC_003075.3) in Arabidopsis, in which the Arabidopsis SPT1 (NC_001036719) is located, it was found that Pt-SPT1 almost showed the same alternative splicing pattern compared with the Arabidopsis SPT1, with only one exon missing in Pt-SPT1 (Figure 4.2 and Table 4.2). Checking the splicing sites in the Arabidopsis chromosome 4, almost all splicing sites follow the GT-AG rule except the second pair in GA-AG pattern. The data also show that the splicing starts from nucleotide 504 and ends at nucleotide 1840 in Pt-SPT1, which matches the ORF region of Pt-SPT1 from nucleotide 471 to nucleotide 1871. These results also demonstrated that the chromosome of Loblolly pine that contains the Pt-SPT1 might be highly conserved in this ORF region. Probably intros and exons presented in pine chromosome are roughly similar to Arabidopsis chromosome 4. However, exons in 3' UTR and 5'UTR region of Pt-SPT1 have different sequences compared with At-SPT1 of Arabidopsis to associate with different transcriptional mediation in pine. Alignment

results between Arabidopsis chromosome4 and Pt-SPT1-4 indicate that one exon is present from nucleotide 406 to 515 in Pt-SPT1-4 with 64.5% sequence identity with the exon11 in At-SPT1. The alignment results among chromosome 4 in Arabidopsis and Pt-SPT1-2 and Pt-SPT1-3 indicate that two exons are present in Loblolly pine, which are identity to the exon 9 and exon 11 in At-SPT1. The conserved SAM domain in both small transcripts spans these two exons. Again, the results reveal that SPT enzyme might be functionally highly conserved through evolution. Further, the chromosome in Loblolly pine, which contains the Pt-SPT1, might be slightly different to the chromosome 4 in Arabidopsis.

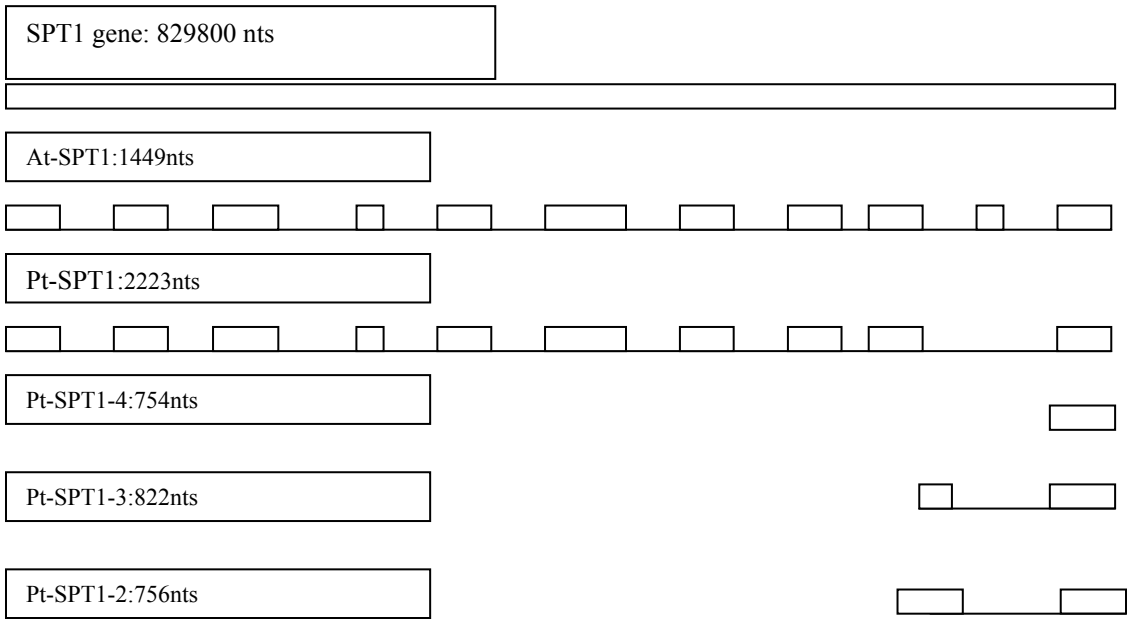


Figure.4.2. Align multiple Pt-SPT1 transcripts with At-SPT1 (SPT1 of Arabidopsis) and chromosome 4 in Arabidopsis
Exon1 to Exon11 from left to right in order.

Table 4.1. Exon (in Figure 4.2) position, length, identity to the chromosome 4 in Pt-SPT1 and At-SPT1 (Arabidopsis SPT1)

Name		Genomic	mRNA	Length	Identity %	Mismatches	Gaps	Donor
At-SPT1	Exon1	1085-1198	88-201	114	93	8	0	d
	Exon2	1323-1414	202-293	92	100	0	0	d
	Exon3	1495-1599	294-398	105	95.2	5	0	d
	Exon4	1678-1730	399-451	53	100	0	0	d
	Exon5	1825-1951	452-578	127	96.1	5	0	d
	Exon6	2090-2279	579-768	190	94.2	11	0	d
	Exon7	2381-2521	769-909	141	100	0	0	d
	Exon8	2608-2703	910-1005	96	100	0	0	d
	Exon9	2796-2937	1006-1147	142	100	0	0	d
	Exon10	3037-3164	1148-1275	128	100	0	0	d
	Exon11	3239-3412	1276-1449	174	100	0	0	d
Pt-SPT1-1	Exon1	1085-1198	504-617	114	58.8	47	0	d
	Exon2	1323-1414	618-709	92	72.8	25	0	d
	Exon3	1495-1599	710-814	105	66.7	35	0	d
	Exon4	1678-1730	815-867	53	75.5	13	0	d
	Exon5	1825-1951	868-994	127	71.7	36	0	d
	Exon6	2090-2279	995-1190	196	63.3	72	6	d
	Exon7	2381-2521	1191-1331	141	73.8	37	0	d
	Exon8	2608-2703	1332-1427	96	64.6	34	0	d
	Exon9	2796-2942	1428-1574	147	66.7	49	0	
	Exon10	3272-3381	1731-1840	110	64.5	39	0	
Pt-SPT1-4	Exon1	3272-3381	604-515	110	64.5	39	0	
Pt-SPT1-3	Exon1	2914-2942	290-318	29	86.2	4	0	
	Exon2	3272-3381	475-584	110	64.5	39	0	
	Exon1	2820-2942	129-251	123	69.9	37	0	
	Exon2	3272-3381	408-517	110	63.6	40	0	

I did a nucleotide alignment of SPT1 genomic and mRNA sequences from Human, Arabidopsis and Drosophila using the Spidey program (http://www.ncbi.nlm.nih.gov/IEB/Research/Ostell/Spidey/). Nucleotide sequences are in the appendixes (see Appendix 5 on page 133). The tables show nucleotide identity and placement of exons relative to the genome sequence. The mRNA sequence cited by GenBank as the Reference RNA (usually the longest sequence) was taken as the reference spliced form. The tables show that 4 out of the 5 Human SPT1 sequences compared were alternatively spliced, 3 out of 4 SPT1 Arabidopsis mRNAs were alternatively spliced and 1 out of 2 Drosophila mRNAs were alternatively spliced

compared to the reference splicing pattern (APPENDIX 4). These results are consistent with my Pine SPT1 clones showing alternative splicing. This indicates that alternative splicing of SPT1 is a general phenomenon.

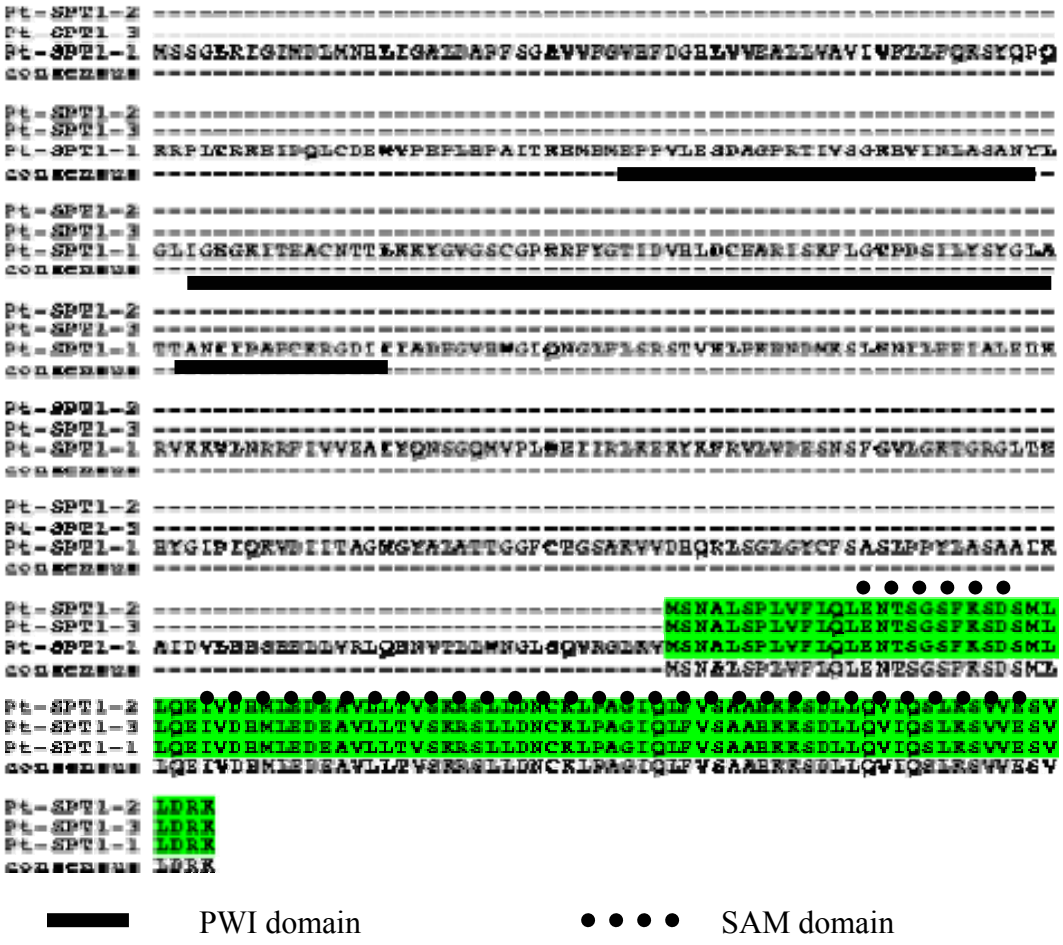
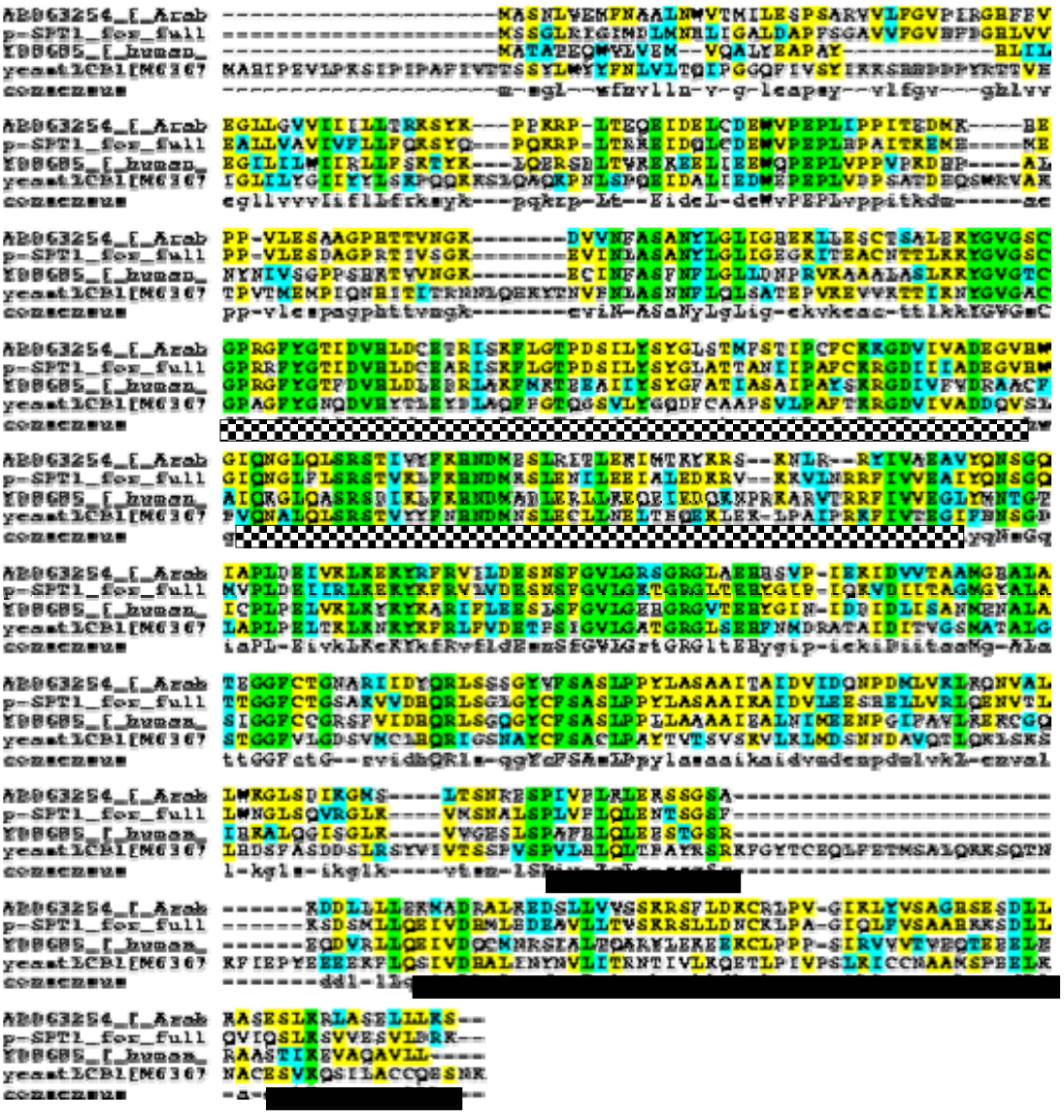


Figure 4.3. Amino acid alignment of predicted proteins encoded by Pt-SPT1-1, Pt-SPT1-2 and Pt-SPT1-3

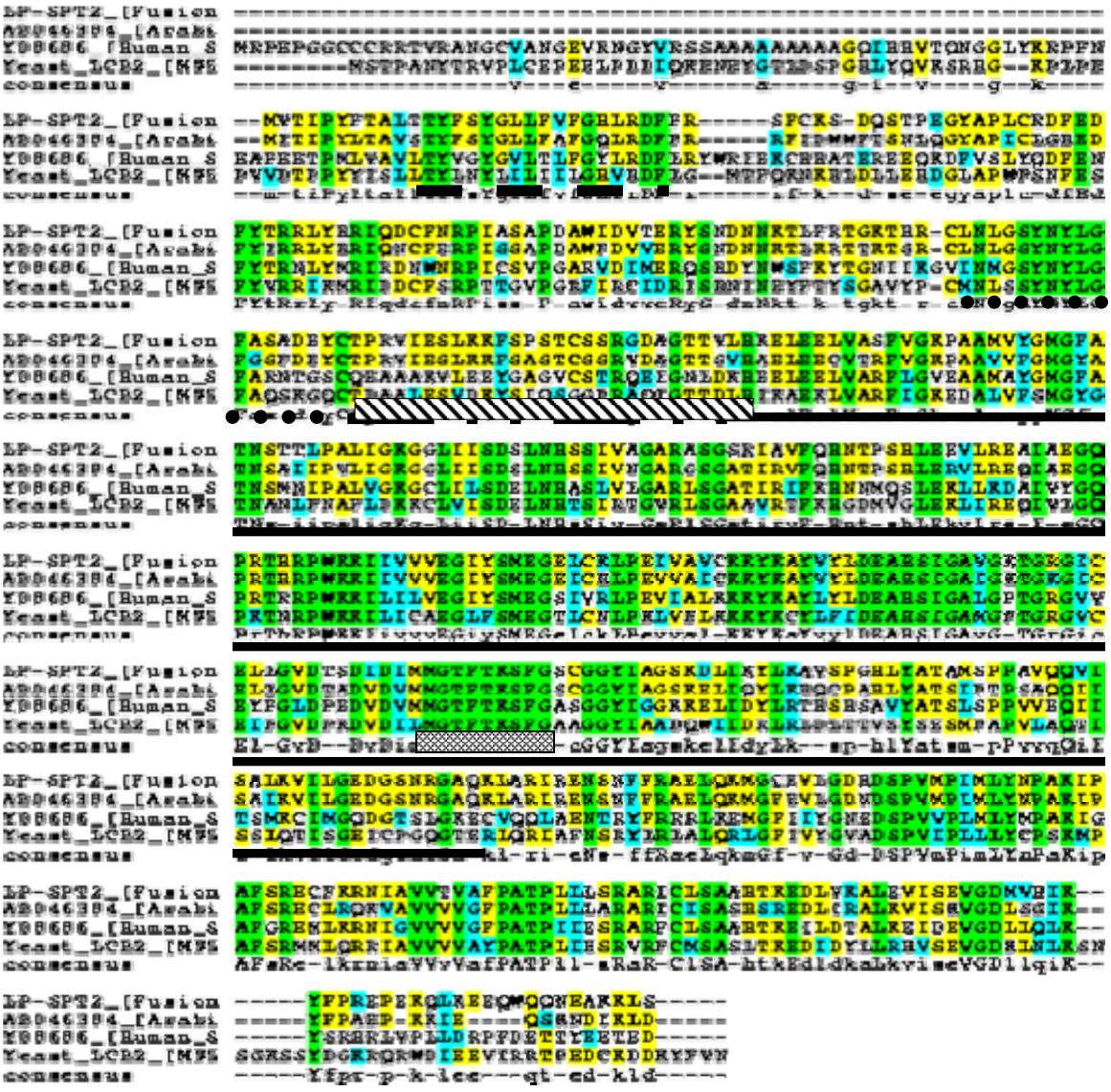
The cloned pine serine palmitoyltransferase subunit 1 (Pt-SPT1-1) 'full length' encodes a protein of 484 a.a. (Figure 4.3) in the regions of overlap the Pt-SPT1 clones are identical. The apparent splice occurs before the initiating methionine in clone Pt-SPT1-1. Both Pt-SPT1-2 and Pt-SPT1-3 encode a 90 amino acid peptide (Figure 4.3) which is in the same frame as the 484 amino acid protein. Pt-SPT1 has 62% identity and 81% similarity to SPT1 in Arabidopsis, and 47% identity and 66% similarity to human SPT1

subunit. Using the same strategy, a cDNA clone for a presumptive serine palmitoyltransferase subunit 2 (Pt-SPT2) was cloned. Pt-SPT2 encodes a protein with 493 a. a. which has 79% identity and 88% similarity to SPT2 in Arabidopsis and has 53% identity and 72% similarity with human SPT2 subunit. Only a single form of the Pt_SPT2 mRNA was found.



Part A : PWI domain SAM domain

Figure 4.4 (Part A). Amino acid sequences alignment of SPT1 sequences in yeast (M63674), Arabidopsis (AB063254), Loblolly pine (p-SPT1) and human (Y08685)
PWI domain: hatched underline; The SAM domain: solid black



Part B: ■ ■ ■ **PH domain** ● ● ● ● **EGF-CA domain** ▨ **HALZ domain** ▬ **CMM domain** ▩ **PLP binding domain**

Figure 4.4 (Part B). Amino acid sequences alignment of SPT1 sequences in yeast (M63674), Arabidopsis (AB063254), Loblolly pine (p-SPT1) and human (Y08685)

B Amino acid sequence alignment of SPT2 Yeast (M95669), Arabidopsis (AB046384), Loblolly pine (LP-SPT2) and human (Y08686). Pyridoxal phosphate-binding motif: hatched underline; PH domain: square dotted line; Cys-Met-Meta-pp: solid black line; EGF-CA domain: circle dotted line; The HALZ domain: stripped line (http://seqtool.sdsc.edu/CGI/BW.cgi).

Pt-SPT1 domain analysis

The predicted Pt-SPT1 and Pt-SPT2 protein were analyzed using the SMART software program (http://smart.embl-heidelberg.de/smart/set_mode.cgi?NORMAL=1) (Figure 4.4). Both proteins contained an aminotransferase domain (Pfam: Aminotran_1_2, E value, Pt-SPT1-1, $1.4e^{-23}$ and Pt-SPT2, $5.7e^{-61}$), Based on the previous study (Mukherjee & Dekker 1990), this is a common domain in pyridoxal-phosphate dependent enzymes, The smaller transcripts Pt-SPT1-2 and Pt-SPT1-3 which encode a 90 a.a peptide, lack this domain.

Table 4.2. Predicted alternative SPT transcripts in various organisms

	SPT1			SPT2			SPT1		SPT2	
	mRNA	Access #	Protein (a. a.)	mRNA	Access #	Protein (a.a)	Location			
Yeast	2,468 1,677	M63674 AY693052	558	2,843 1,686	M95669 P40970	561	3 TMD	No TMD	ER MS	ER MS
		CAA18397	508							
Arabidopsis	1,813 2,098 1,952 1,540	AB063254 AY120759 NM_001036719 AAN15450	482	1,973 1,501 1,593 2,014 2,072	AB046384 AY133827 AB074928 AY050829 NM_12272	489	NO TMD	TMD 2-24	ER MS	ER MS
	1,428	CAB80314	475							
Human	2,742	AAH68537	513	2,026 7,250	Y08686 NM004863	562	TMD	TMD	ER MS	ER MS
	1,621 2,780	Y08685 NM006415	473	3,855	NM_018327	552				
	998 973	NM_178324 AAH07085	143	4,828	EAW81298	470				
	195	XP_001130723	64		EAW68374	685				
Drosophilla	1,407 1,591 1,521	NP_725256 NM_165967 NM136998	468	1,794	BAA83721	597	TMD 20-37	NO TMD	ER MS	ER MS

To predict the sub-cellular location of the putative serine palmitoytransferase in Loblolly pine, the TMHMM program in Biology WorkBench (http://seqtool.sdsc.edu/CGI/BW.cgi#!) was used. The results showed that both Pt-SPT1 and Pt-SPT2 had highly hydrophobic domains in their N-terminus, which are likely to be transmembrane. In human, SPT1 and SPT2 both have TMDs (Hanada et al. 1997, Batheja et al. 2003); Yeast has three TMDs in SPT1 (Han et al. 2004). The same software program (TMHMM) was used to predict the TMD region in SPT1 and SPT2 subunits in human, Arabidopsis and yeast, the results are shown in Table 5.3. The SPT subunits in human, Arabidopsis, and yeast all have larger transmembrane regions than that of SPT in Loblolly pine (prediction data not shown here). These results are consistent with previous results (Hanada et al. 1997, Batheja et al. 2003).

Pt- SPT1 has a PWI domain from residue 152 to residue 255 in (Figure 4.3 A). PWI domains are found in eukaryotes at the N terminal of a number of RNA processing enzymes (Szymczyna 2003) and may be important for protein interactions within splicing complexes (Szymczyna 2003). By aligning amino acid sequences of the SPT1 subunits in Loblolly pine, Arabidopsis and human, the results showed that the PWI region was highly conserved (Figure 4.4 A). However, no report demonstrated the function of the conserved PWI like region in SPT1 in human or Arabidopsis. Functional study of PWI domain by Szymciyna et al demonstrated that the PWI motif in SRm160 splicing and 3' end cleavage-stimulatory factor was a new member in RNA/DNA-binding domains (Szymczyna 2003). This particular motif has an equal binding ability for DNA and RNA (Szymczyna 2003). Deletion of the PWI motif eliminates the capability of SRm160 to bind RNA to stimulate 3' end cleavage, RNA binding can be restored by recombinant

DNA methods, whereby PWI is replaced by a heterologous RNA-binding domain (Szymczyna 2003). The PWI motif in Pt-SPT1 might be involved in the pre-mRNA splicing of the multiple SPTI transcripts of SPT1.

A sterile alpha motif (SAM) domain is present in Pt-SPT1 (409-476) (Figure 4.3 A). The SAM domain is found in diverse proteins, such as receptor tyrosine kinases and transcription factors (Ponting 1995). SAM domains can bind to other SAM-containing protein and, once phosphorylated, to SH2 domains (Shultz et al. 1997). The SAM domain of the Smaug protein helps to generate a morphogen gradient in Drosophila embryos by binding to the 3' UTR of *nanos* (nos) mRNAs and repressing their translation (Aviv et al. 2003). These results suggest that the SAM domain plays a role in RNA recognition and binding. The SAM domain in Pt-SPT1 might work in concert to the PWI motif of Pt-SPT1 in RNA or DNA binding to function in alternative splicing or pursuing a post-transcriptional regulation to control the SPT protein level during embryogenesis of Loblolly pine.

The smaller SPT transcripts in Loblolly pine, Pt-SPT1-2 and Pt-SPT1-3, each encodes an identical 90 a. a. protein which contains a SAM domain (Figure 2). Neither predicted protein contains a Nucleus Localization Sequence (NLS), thus the 90 a. a protein might locate in cytosol. Recently, research by Barrera et al indicated that the SAM domain in P73, a cancer repressor, had lipid binding properties towards an anionic lipid (PA) and a zwitterionic lipid (PC) (Barrera et al. 2003). Thus production of short proteins via alternative splicing may be a mechanism of regulating both lipid metabolism and gene expression at a post transcriptional level.

Pt-SPT2 domain analysis

Pt-SPT2, contains a Cys-Met-Meta-pp (CMM) domain from the residue 133 to residue 375 (Figure 5.3B, in solid black). This CMM domain is involved in cysteine and methionine metabolism, and is common in the α-oxoamine synthase subfamily of which SPT is a member (Clausen et al. 1996). While the members in this subfamily catalyze a range of related reactions they all use PLP (pyridoxal-5'-phosphate) as a cofactor (Clausen et al. 1996). PLP is a cofactor in the SPT catalyzed condensation of L-serine and palmitoyl coenzyme A to form 3-ketodihydrosphingosine. SPT have a highly conserved PLP binding motif, GTFTKSFG motif, which was also found in Pt-SPT2 (Figure 4.4 B).

The Calcium-binding EGF-like domain EGF-CA, is present from residue 99 to 120 of Pt-SPT2 (Figure 4.4 B).This EGF-like domain contains totally 22 residues (Figure 4.4 B). Among these 22 residues, 7 of them (SYNYLG) are exactly conserved through yeast, to Arabidopsis, Loblolly pine, and human. The EGF-CA motif is found in many membrane-bound and extra cellular animal proteins which require calcium for catalytic function and/or protein-protein interaction (Stenflo et al. 2000).

The Homeobox Leucine Zipper (HALZ) domain is plant specific and is a common DNA-Binding motif in transcription factors (Schena & Davis 1994). A HALZ domain is found in Pt-SPT2 from the residue 120 to residue 166 in Pt-SPT2 (Figure 4.4 B). Amino acid alignment shows that this domain is almost identical in the SPT2 protein of Loblolly pine and Arabidopsis with only two residues difference.

The discovery of multiple SPT1 transcripts in Loblolly Pine embryos encoding alternative forms of SPT proteins and further search for similar sequences in other

organisms (Table 4.3) reveals numerous variants of SPT1 in humans, Arabidopsis, yeast and Drosophila and found some are alternative splicing results. In most cases BLAST yielded a single SPT2 protein sequence, however in humans a number of SPT2 protein sequences were found.

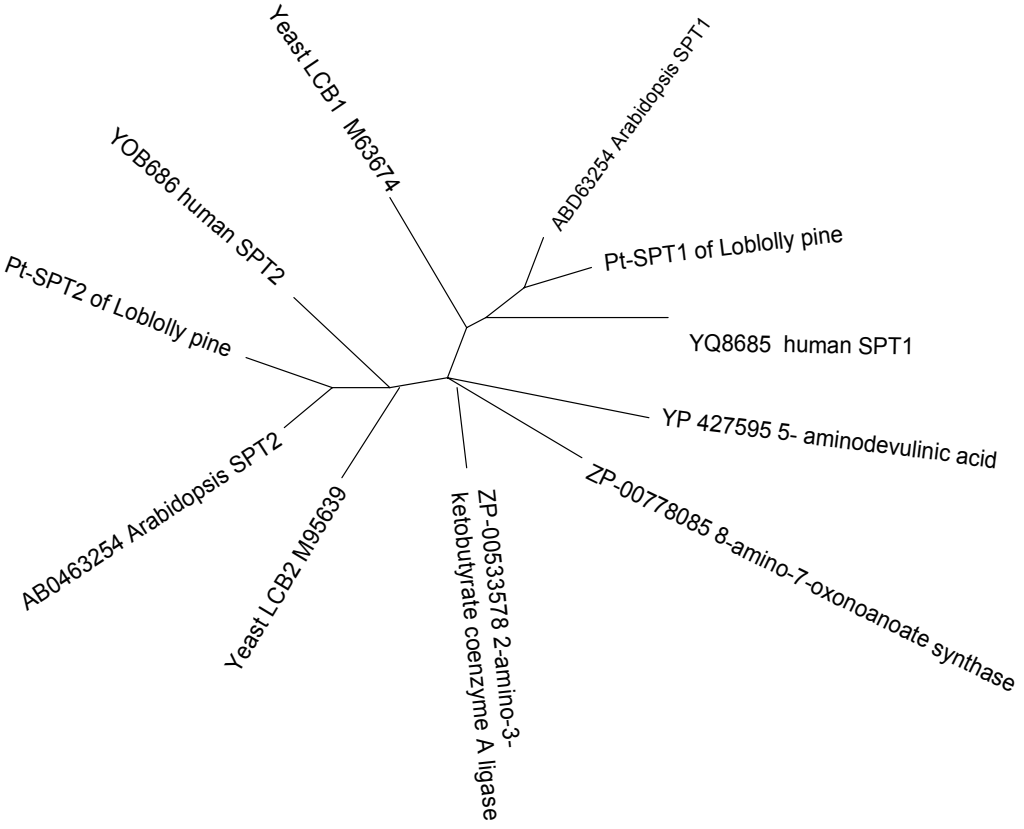


Figure 4.5. Phylogenetic relationships among members of the POAS family determined by multiple alignments using the ClustalW algorithm
There are three subgroups: Group 1 includes: Yeast LCB1 (M63674), Arabidopsis SPT1 (AB063254), Human SPT2 (Y08685) and Loblolly pine SPT2. Group 2 includes: Yeast LCB2 (M95669), Arabidopsis SPT2 (AB046384), Human SPT2 (Y08686) and Loblolly pine SPT2. Group 3: 5-aminolevlinic acid synthase (YP-427595), 2-amino-3-ketobutyrate synthase (ZP-00533578), 8-amino-7-oxononanoate synthase (ZP-00778485).

4.3.2. The evolutionary position of Pt-SPTs in Loblolly pine

Phylogenetic analysis shows Pt-SPT1 and Pt-SPT2 are highly conserved throughout evolution. All the subunits of SPT1 from yeast to plants then to human are found in a closely related subgroup. SPT2 is aso highly conserved and widely expressed during evolution, (Figure 4.5). All the pyridoxal 5'-phosphate dependent α-oxoamine synthases (POAS) like enzymes belongs to the super-family, POAS. Through evolution, SPTs eventually evolved into two distant sub-groups: the SPT1 subgroup and SPT2 subgroup.

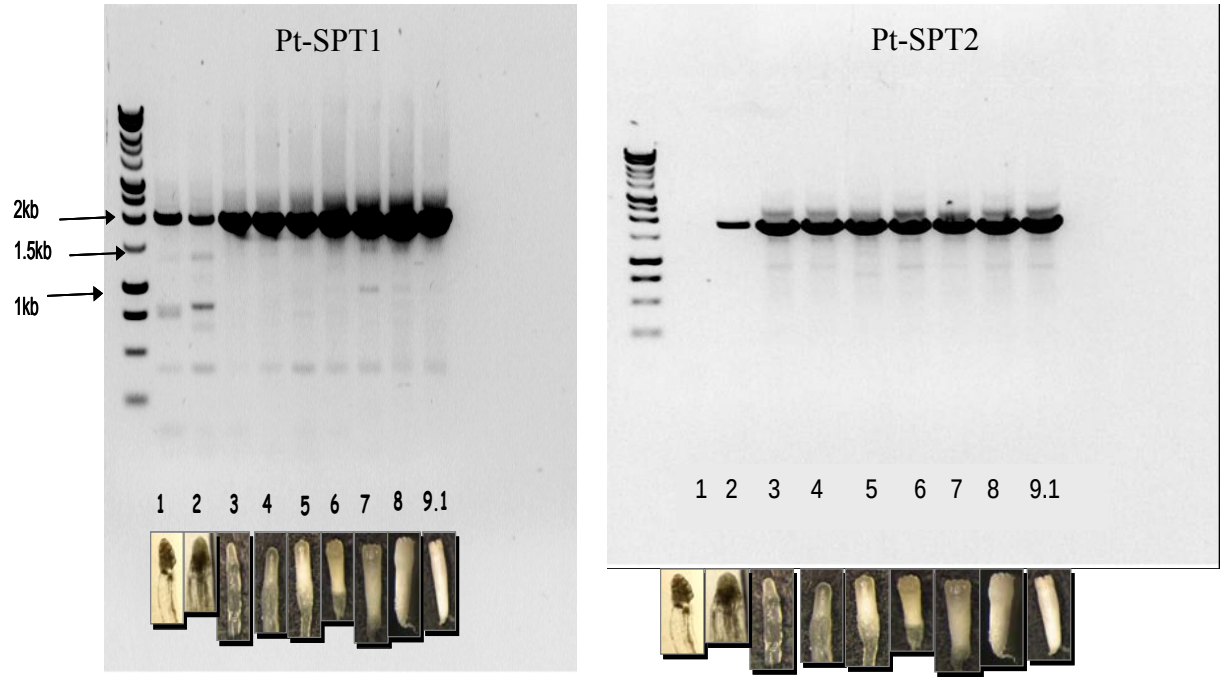


Figure 4.6. Expression of Pt-SPT1 and Pt-SPT2 in zygotic embryos assayed by semi-quantitative PCR
Primers specific to Pt-SPT1 and Pt-SPT2.were used to monitor mRNA levels in zygotic embryos of Loblolly pine from stage (1-9.1)

4.3.3. Expression pattern of SPT in Loblolly pine during embryogenesis

To study the Pt-SPT expression pattern in zygotic and somatic embryogenesis process, semi-quantitative PCR was conducted. Identical amounts (210 ng) of total RNA were used for cDNA synthesis. Conventional controls such as monitoring the level of actin mRNA have proven inexact since the actin family is large in pine. Recently primers have been designed that are successful for pine actin mRNA, (Cairney personal communication) however these and the necessary protocols were not available to me at the time. The cDNA created and used in these experiments has been used by others in the laboratory and consistent, verifiable results have been obtained. For this reason, while the lack of a control transcript limits interpretation, there is good reason to believe that the general trends that were observed are accurate. Levels of Pt-SPT1 mRNA apparently rise from stage 1 to the mature stage 9.1 (Figure 4.6). After stage 9.1, mRNA for this subunit remains steady at this level all the way to the completion of embryo maturity, stage 9.10 (data not shown). By contrast, Pt-SPT2 mRNA is undetectable in early embryo stage 1 and stage 2, increases rapidly at early stage 3 and, then remains at a constant level till the embryo matures (Figure 4.6).

Distinct expression patterns of Pt-SPT1 are displayed in zygotic and somatic embryos (Figure 4.7). In contrast to the gradually increasing of Pt-SPT1 expression in zygotic embryos, Pt-SPT1 mRNA is present at a constant amount through early stages to the late stage in somatic embryogenesis. In each stage, the amount of Pt-SPT1 expression in zygotic embryos is much higher than the expression amount of Pt-SPT1 in somatic embryos (for visualization, purposes, three fold more RT-PCR products were loaded for somatic embryos than for zygotic embryos). Because the principal Pt-SPT1 would be

expected to play critical roles in sphingolipid regulation, failure to produce it in a normal level might affect somatic embryo development.

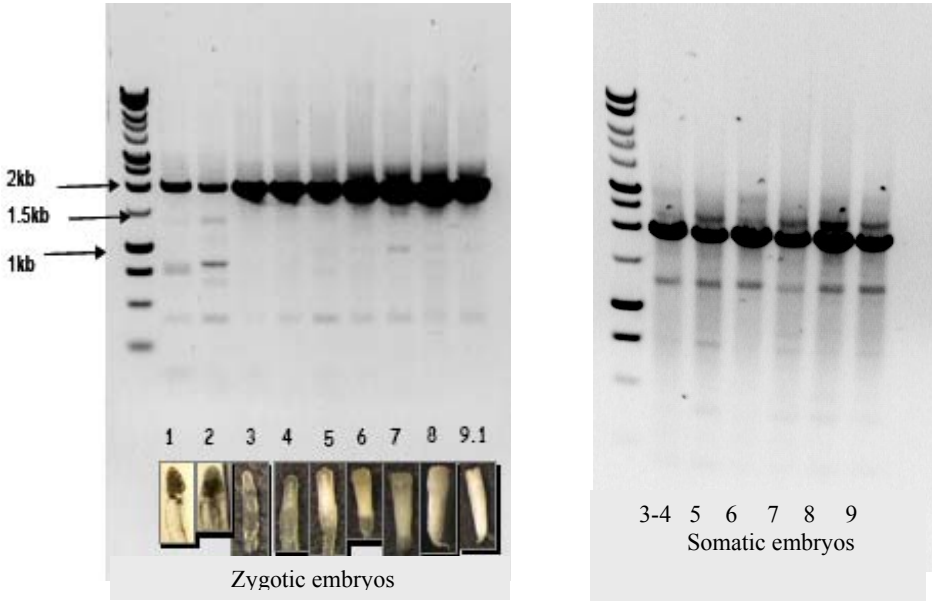


Figure 4.7. Products of semi-quantitative PCR using primers specific to Pt-SPT1
Pt-SPT1 expression pattern in zygotic embryos (left) and in somatic embryos (right)

4.4. Conclusions

Many of the enzymes that have been characterized to date in yeast and Arabidopsis appear to be represented by ESTs in the Pine Gene Index. Further Detailed sequence analysis of Loblolly Pine cDNAs showing strong similarity to Pt-SPT1 and Pt-SPT2 in Arabidopsis, yeast, and human suggested that SPT proteins would exhibit similar catalytic and regulatory activities to SPT enzymes that have been characterized.

The most intriguing finding was the existence of multiple transcripts for Pt-SPT1, all of which seemed capable of producing a protein. These proteins contained important

functional domains, thus the shorter proteins could act as Dominant Negative Mutant proteins or have independent functions. We searched the databases and found similar instances of apparently spliced SPT transcripts which would produce alternative SPT proteins, in yeast, Arabidopsis, Drosophila and Human. Although little is known about the existence of alternative spliced form of SPT mRNA being expressed as SPT isoenzymes, recent studies by Jia Wei and colleagues in the Merrill laboratory at Georgia Tech (personal communication) have found an SPT1 isoform with an alternative C-terminal 40 a.a. that is detected by antibody to the unique sequences. These, presumptive, novel SPT proteins add to the complexity of sphingolipid regulation and to the discussion of the nature of the functional SPT enzyme (Hornemann et al. 2006).

CHAPTER 5: Characterization and expression analysis of ceramide kinase genes in Loblolly pine

Abstract

Ceramide kinase (CERK) was investigated in loblolly pine. Two different versions of mRNAs, with nucleotide 2786 bps (Long), and 2320 bps (Short) respectively, of ceramide kinases in loblolly pine (Pt-CERKs) have been cloned. Long version encodes a protein with 721 amino acids, the short version has 560 amino acids. Short chain CERK has 431 nucleotides fewer compared to Pt-CERKL, thus the Pt-CERKS protein possesses a catalytic domain but lacks three motifs: a ZnF-C4 domain and a Ca^{+2} calmodulin-like binding motif and a critical residue, glycine. The expression patterns for these two versions are different through embryo development. Long version is constitutively expressed, while the short one is only expressed in some stages. These cDNAs have been cloned into expression vectors to produce Pt-CERKL and Pt-CERKS proteins in *E.coli*, The purified Pt-CERKL and Pt-CERKS proteins, were then analyzed for kinase activity in vitro. The results suggested that these two Pt-CERKs have similar catalytic functions as their homologus in human and Arabidopsis, to catalyze the formation of ceramide-1-phosphate from ceramide. But, even a short peptide chain with 399 a. a, which contains the DAGK domain, appears to have catalytic activity. With regard to dependence on divalent cation, Ca^{2+}, three different Ca^{2+} concentration buffer were prepared for enzyme assay, and the results suggest that the Loblolly pine CERKs were less dependent on Ca^{2+} ions. The presence of a membrane system does not appear to be necessary for the Pt-CERKs activity in vitro.

5.1. Introduction

Sphingolipids are well known as important cell membrane components (Merrill 2002). It has also been found that they also play very important roles as signal molecules in cell growth, cell death, and in embryogenesis of animals (Bird & Kimber 1984; Merrill & Sandhoff 2002; Kudo et al. 2004). Ceramide (Cer), which is the precursor of all complex sphingolipids and small active sphingolipids, is found to be involved in cell cycle arrest and cell apoptotic pathways (Obeid et al. 1993; Perry & Hannun 1998). A sphingolipid metabolism enzyme, known as ceramide kinase (C1P), which is an ATP dependent kinase, converts ceramide to ceramide 1-phosphate which reduces the cell death-inducing effects of ceramide (Hinkovska-Galcheva et al. 1998; Gijsbers et al. 1999; Sugiura et al. 2002; Liang et al. 2003)**.** Besides this mitogenic effect, CERK is also involved in two other two biologic activities: the fusogenic activities and the cytoprotective activities in both animal and plants. For example, ceramide kinase was reported to be closely related to phagocytosis in animals and defense reactions in plants (Liang et al. 2003; Lamour & Chalfant 2005; Lamour et al. 2007). C1P was characterized as a novel signaling lipid participating in eicosanoid synthesis to promote release of arachidonic acid and PGE2 in A549 human lung carcinoma cells by binding to the cytosolic phosphalipase A2 (Pettus & Bielawska 2004; Chalfant & Spiegel 2005). C1P was also found to be a potential fusogen and appears to play a role in the activation and degranulation of RBL 2H3 rat basophilic leukemia cells (Hinkovska-Galcheva 1998). Research by Mitsutake (Mitsutake & Igarashi 2005) demonstrated that ceramide kinase and ceramide-1-phosphate were required for the deregulation process in mast cells and that this process was dependent on calmodulin (Mitsutake & Igarashi 2005). Another

study on GH4C1 rat pituitary cells showed that C1P can stimulate calcium entry through voltage-operated calcium channels (Ottico et al. 2003). Therefore, ceramide kinase is one of the conversion points in controlling the balance between ceramide and ceramide 1-phosphate in changing cell fate (from cell death and cleaning to cell survival) and regulating cell physiological activities. Ceramide kinase (CERK), like the sphingosine kinase and diacylglycerol (DAG) kinase, belongs to the lipid signaling kinase superfamily (Wattenberg et al. 2006). CERKs are present in diverse multicellular organisms, such as plants, insects, nematodes, and vertebrates; but no homolog gene is identified in yeast so far (Sugiura et al. 2002; Liang et al. 2003).

In animals, ceramide kinase was first identified as a Ca^{2+} inducing kinase in brain synaptic vesicles (Bajjalieh et al. 1989; Kolesnick & Hemer 1990). A human ceramide kinase (*h*CERK) was cloned and proved to phosphorylate ceramide substrates with much higher efficiency than the related sphingosine substrates (Sugiura et al. 2002). The *h*CERK encodes a protein with 537 amino acids, which has a catalytic region with strong similarity to the diacylglycerol kinase catalytic domain.

In plants, based on the NCBI database there are two version CERKs in Arabidopsis, one codes a CERK protein with 608 a. a. (AAQ62904, GI: 34223322) and the other codes a CERK protein with 533 a. a. (BAA97392, GI: 8843866). Arabidopsis (608 a. a.) CERK mutant acd5, which changed glycine to arginine at residue 412, develops normally, initially, but expresses excessive cell death later when infected with bacterial pathogen *Psudomonas syringae* (Liang et al. 2003). Research also showed that the later cell death of this mutant Arabidopsis plant is largely dependent on external stress and defense

singling, which is controlled by the plant hormone ethylene and the phenolic salicylic acid respectively (Greenberg et al. 2000).

However, a couple of contradictions are present in reports about ceramide kinase. For example, regarding subcellular distribution, ceramide kinase, which was first identified in brain synaptic vesicles and was known as a membrane-associated protein (Bajjalieh et al. 1989; Kolesnick & Hemer 1990). Mitsutake et al (Mitsutake & Igarashi 2005) recently it was indicated that *h*CERK was originally localized in the cytosol of RBL-2H3 cells and present later at the plasma membrane in a few cells. However, Carre et al reported a Golgi localization in Cos-1 and HUVEC cells, and the *h*CERK could be translocated to the plasma membrane upon osmosis (Carre et al. 2004). Regarding the calcium dependence of the catalytic reaction of ceramide kinase, van Overloop demonstrated that bacterially expressed *h*CERK was highly dependent on Mg^{2+} and showed much less dependence on Ca^{2+} ions (van Overloop et al. 2006). However, Mitsutake et al found that calmodulin was involved in the Ca^{2+} -dependent activation of CERK (Mitsutake & Igarashi 2005), thus Ca^{2+} ion are still important for CERK function.

CERK has been studied in mammals, and in Arabidopsis; however, there is less understood about CERK and its functions in pine. Since somatic embryogenesis is a process in which embryos develop under abnormal conditions and since CERK activity is central to healthy development, it is interesting and necessary to investigate the function of ceramide kinase during Loblolly pine embryo development. Accordingly, it is assumed that ceramide kinase has the same function in Loblolly pine as its orthologs in other plants or mammals in controlling the ceramide level and mediating the active sphingolipid, C1P, during embryo development of Loblolly pine. Thus, the investigations

in this study were directed towards characterizing CERK activity during Loblolly pine embryogenesis.

5.2. Materials and methods

5.2.1. Materials

Pine cones were harvested weekly from open-pollinated Loblolly pine (*Pinus taeda* L.) 7-56 mother trees; collections occurred from June 20th to September 15th in 2004 and 2005 in a commercial pine orchard in Lyons, Georgia, US. Early stage embryos were isolated from seeds, dissected and embryos were removed and immediately flash frozen in liquid N_2, then stored at -70 ^{o}C prior to use. The staging system to determine Loblolly pine embryo development has been described previously (Pullman and Webb 1994, Ciavatta et al. 2001). Somatic embryo cell lines were initiated from immature megagametophytes isolated from Loblolly pine seed. Cells were sub-cultured weekly. Maturation of somatic embryos was achieved using media and methods of Pullman et al (Pullman et al. 2003). NBD labeled C6-ceramide, TLC plates and ATP were generously provided by Dr. Al. Merrill's lab (School of Biology, Georgia Institute of Technology). BD SMART Race Amplification Kit is purchased from BD Biosciences. DNA polymerase, JM 109 competent cells, PCR, restriction enzymes (EcoR I), and gel purification kits were obtained from *Promega*. NheI was purchased from New England Nuclear. The 5ml TALON® Single Stem Columns and related reagents for protein purification were purchased from *Clontech*. pET28a+ vector and expression host cell DE3 cells were purchased from *Novagen*. All other chemicals were purchased from *Sigma*.

5.2.2. Clone full length Pt-CERKs

Total RNA was isolated from chosen stage 7 and stage 9.1 embryos using RNeasy Mini Kit from *QIAGEN* (Qiagen 2006) with the procedure described in this kit manual. The sequences of pine homologs (TC45250, EST in the Pine Gene Index: http://compbio.dfci.harvard.edu/tig/cgi-bin/tgi/gimain.pl?gudb=Pine) of ceramide kinase (AY362552) in Arabidopsis were used to design the gene specific primers by *Primer* 3 software program (http://frodo.wi.mit.edu/) to get 3'RACE Gene specific primer for ceramide kinase in Loblolly pine (5'-CCAATCCGAGATCGGGATGTGAT-3') and the 5'RACE Gene specific primer for ceramide kinase in Loblolly pine (5'-GAACCCCCACTAGCAAGCTCCACAGCA-3') for the 5'-RACE-Ready cDNAs and 3'-RACE-Ready cDNAs .

BD SMART RACE Kit included a protocol for the synthesis of two separate cDNA populations: 5'-RACE-Ready cDNA and 3'-RACE-Ready cDNA. The cDNA for 5'-RACE was synthesized using a modified lock-docking oligo (dT) primer and the BD SMART II AN oligo. The modified oligo (dT) primer, termed the 5'RACE CDS Primer (5'-CDS), had two degenerate nucleotide positions at the 3'end (Barnes 1994). Follow the kit manual for detail procedures (Qiagen 2006).

Design the forward and reverse primers based on the 5' cDNA sequence and 3'cDNA sequence as following: Pt-CERKF (5'-AGAGGGCGGGGACGTCCATCCA-3'); Pt-CERKR (5'-TGTGCGCTTCCCCTCCCAATA-3'). Use this pair of primers to run PCR to get full length Pt-CERKs.

Use pGEM-T Easy Vector Systems (*Promega*, Madison, WI) to do the ligation. Use the pGEM-T Easy Vector Ligation Reaction to transform pGEM-T Easy vector to amplify Pt-CERKs fragments.

5.2.3. Construction of expression vector

Gel electrophoresis, restriction enzyme digestion, ligation of DNA and transformation of *E.coli* were performed according to the protocols described in the reference (Sambrook 2001). The CERK long version (Pt-CERKL) and CERK short version (Pt-CERKS) full-length cDNAs were obtained by PCR amplification on the plasmid pGEM T Easy vector with primers Pl-F-CERK(5'-CTAGCTAGCTTCGAAGGTAACGTTCTGAGCAACAA3'); Pl-R-CERK (5'-CCGGAATTCTTGGAGGTGACCCCGGATTTAAACA-3'); A short fragment of Pt-CERKS (Pt-CERKF) was obtained by PCR amplification on the stored plasmid pGME T Easy vector with primers Ps-F-CERK (5'- CTAGCTAGCGAGGGGGAAAGACCCCCGTCTAA-3') and Ps-R-CERK (5'- CCGGAATTCTGATGGATTCAGGAAAGGAGGGCATAG-3'). Use above primers, a NheI restriction site was introduced to the 5'-end of Pt-CERK cDNA sequences and an EcoRI restriction site was introduced to the 3'-end of Pt-CERK cDNA sequences. A 1798 bps DNA (Pt-CERKL) fragment; a 1385 bps DNA (Pt-CERKS) fragment and a 1218 bps fragment of Pt-CERKS (Pt-CERKF) were produced with above primers. PCR fragment Pt-CERKs were purified by agarose gel electrophoresis, digested with NheI/EcoRI, and ligated into the NheI/EcoRI sites of pET28a+ to produce plasmid pET28-Pt-CERKL, pET28-Pt-CERKS and pET28-Pt-CERKF. The final recombinant plasmids were characterized by restriction enzyme digestion and regions around the cloning sites was re-sequenced to ensure that the desired constructions were prepared correctly.

5.2.4. Expression and purification of Pt-CERK proteins

The recombinant plasmids were transformed into *E.coli* strain BL21 for large-scale expression. Cells were grown at 37 ^{o}C with shaking in 2% LB broth (pH 7.4). When the turbidity (A_{600}) reached 1.0, IPTG (100 mM) was added to get 0.4 mM IPTG concentration and 0.8 mM IPTG concentration, respectively, growth was continued at 18 ^{o}C overnight and then the cells were harvested.

Use 5 ml TALON® Single Step Column Purification (BD Clontech, Mountain View, CA). Follow the detail procedures described in the TOLON Mental Affinity Resins User Manual (Company 2006).

5.2.5. Pt-CERKs activity assay

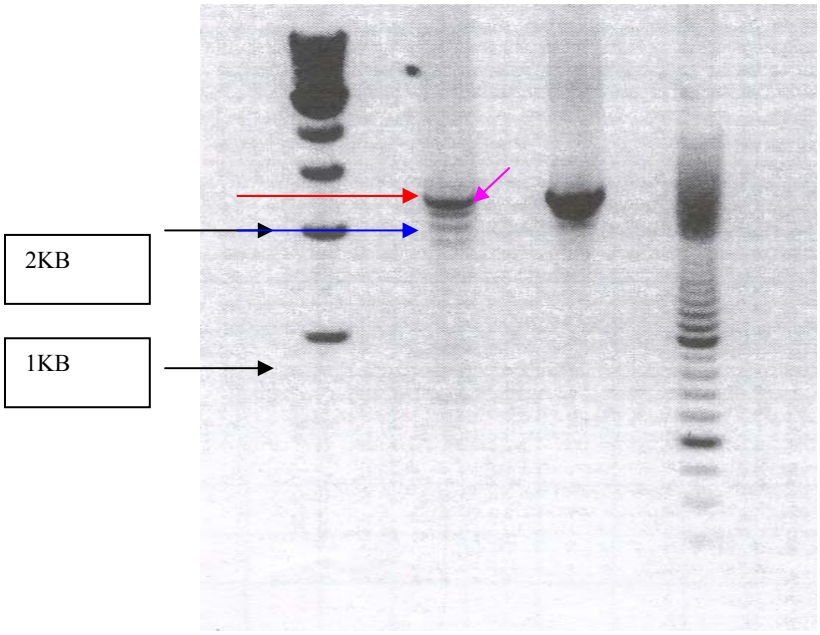
Ceramide kinase activity was measured according to published protocols (Bajjalieh 1989). This was performed in total volumes of 100 μl by mixing 20 μl substrate NBD-C6-Ceramide micelles with 20 μl 5X reaction buffer (75 mM MOP, pH 7.2, 250 mM NaCl, 15 mM $CaCl_2$, 2.5 mM dithiothreitol (DTT), 5 mM EGTA, pH 7.5), 54.9 μl purified Pt-CERK proteins. The reaction was initialed with addition of 10 μl of 5 mM magnesium, 5 mM ATP and was incubated at 37 ^{o}C for 25 minutes. Reactions were stopped by adding 1.2 ml of chloroform/methanol (1:1 as v/v). Phases were broken by adding 530 μl of 1 M KCl, 20 mM MOPS, pH 7.2, vortexed 30s, followed by 5 min centrifugation, 200 μl of the organic phase was moved and then lipids were extracted and separated on Silica Gel 60 high performance TLC plates using chloroform/methanol/water (25:20:1.1) as the solvent system. Bands corresponding to C1P were quantified using an imaging analyzer BAS2500 (Fuji Film).

A few alternation reactions were carried to test Ca^{2+} effects in different Pt-CERK enzymes by preparation of different $CaCl_2$ concentration: 0 mM, 1.875 mM and 15 mM, of the 5X reaction buffer.

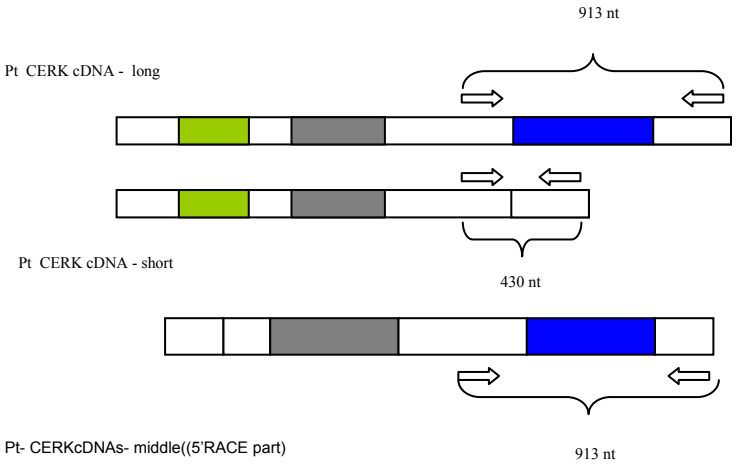
5.3. Results and discussion

5.3.1. Cloning of cDNAs encoding ceramide kinase

In order to clone and study ceramide kinase in Loblolly pine, gene specific primers were designed based on the EST in pine in the Pine Gene Index <http://compbio.dfci.harvard.edu/tgi.cgi-bin/tgi/gimain.pl?gudb=pine>, which is the homolog of Arabidopsis CERK. Specific primers were designed as 5' primer: 5'-TGCAGGAAAAACCTCGCCGTCCAC-3' and 3'primer: 5'-ACCCAATCCGAGATCGGGATGTGATAA-3'; then the 3'RACE and 5' RACE fragments were obtained using BD SMART Race Amplification Kit (BD Clontech, Mountain View, CA). Based on the 3'RACE and 5' RACE fragments, gene specific primers in the 5'UTR and 3'UTR parts were designed to generate a full length cDNA clones (forward and reverse primers: 5'-AGAGGGCGGGGACGTCCATCCA-3' and 5'-TGTGCGCTTCCCCTCCCAATA-3'). Several fragments of different sizes were obtained by PCR (Figure 5.1A) and two different versions of full length cDNAs of ceramide kinase were cloned from zygotic embryos of Loblolly pine (Pt-CERKs), with 2,786 bps, and 2,320 bps, nucleotides respectively. A third cDNA, of intermediate size was also found to be present in Loblolly pine. Efforts to isolate the potential full length version were unsuccessful (Figure 5.1B).



(A)



(B)

Figure 5.1. Cloned two full-length ceramide kinase (Pt-CERKL and Pt-CERKS) and a potential middle size Pt-CERKM

(A) Colored arrows are used to indicate CERK cDNAs of different sizes. The red, 2786bps, blue, 2320bps, between them is the middle size in pink arrow. **(B)** The DAGK (grey) is the conserved catalytic domain, which exists in all three versions. The blue color fragment is lost in Pt-CERKS, while the green rectangle is the PH domain, which is lost in the middle size Pt-CERKm. The blue region lacks in short version.

The long version (Pt-CERKL, 2786 bps) of ceramide kinase in Loblolly pine encodes a protein with 721 a. a., which has 42% identity and 56% similarity to ceramide kinase in Arabidopsis (gene ID:83520) and has 34% identity and 47% similarity to ceramide kinase in human (gene ID:64781). The short version (Pt-CERKS, 2320 bps) encodes a protein with 560a.a. Pt-CERKS has 36% identity and 49% similarity to cermide kinase in Arabidopsis (gene ID: 83520) and has 36% identity and 53% similarity with ceramide kinase in human (gene ID: 64781). Interestingly, the short version lacks 431 nucleotides in the middle part (Figure 5.1 and Figure 5.2). Subsequent examination of public (NCBI) database, different size transcripts of ceramide kinase in Arabidopsis and human were identified**.** Two transcripts in Arabidopsis encode two proteins with 608 a. a. and 533 a. a., respectively (Table 5.1). Aligned these two transcripts have a similar alignment pattern compared to the Pt-CERK cDNAs (shown in Figure 5.2, Figure 5.3). The multiple transcripts look like alternative splicing results. The 5' RACE fragment for the potential middle version (5'Pt-CERKM) encodes a protein with 512a.a (Figure 5.3); 180 nucleotides are absent in the 5'RACE fragment compared with the Pt-CERKL (Figure 5.3). The middle cDNA band is about 2,600 bps, which matches the size of the contiguous sequence formed by fusing the sequence of the overlapping 5'RACE and the 3' RACE cDNA frgments. The cloned 5'RACE fragment, which has only 1,700 pbs, has 100% sequence identity to the fused Pt-CERKM. So it is most likely that a potential middle sized version of the CERK mRNA is represented by the Pt-CERKM with a sequence length about 2,600 bps.

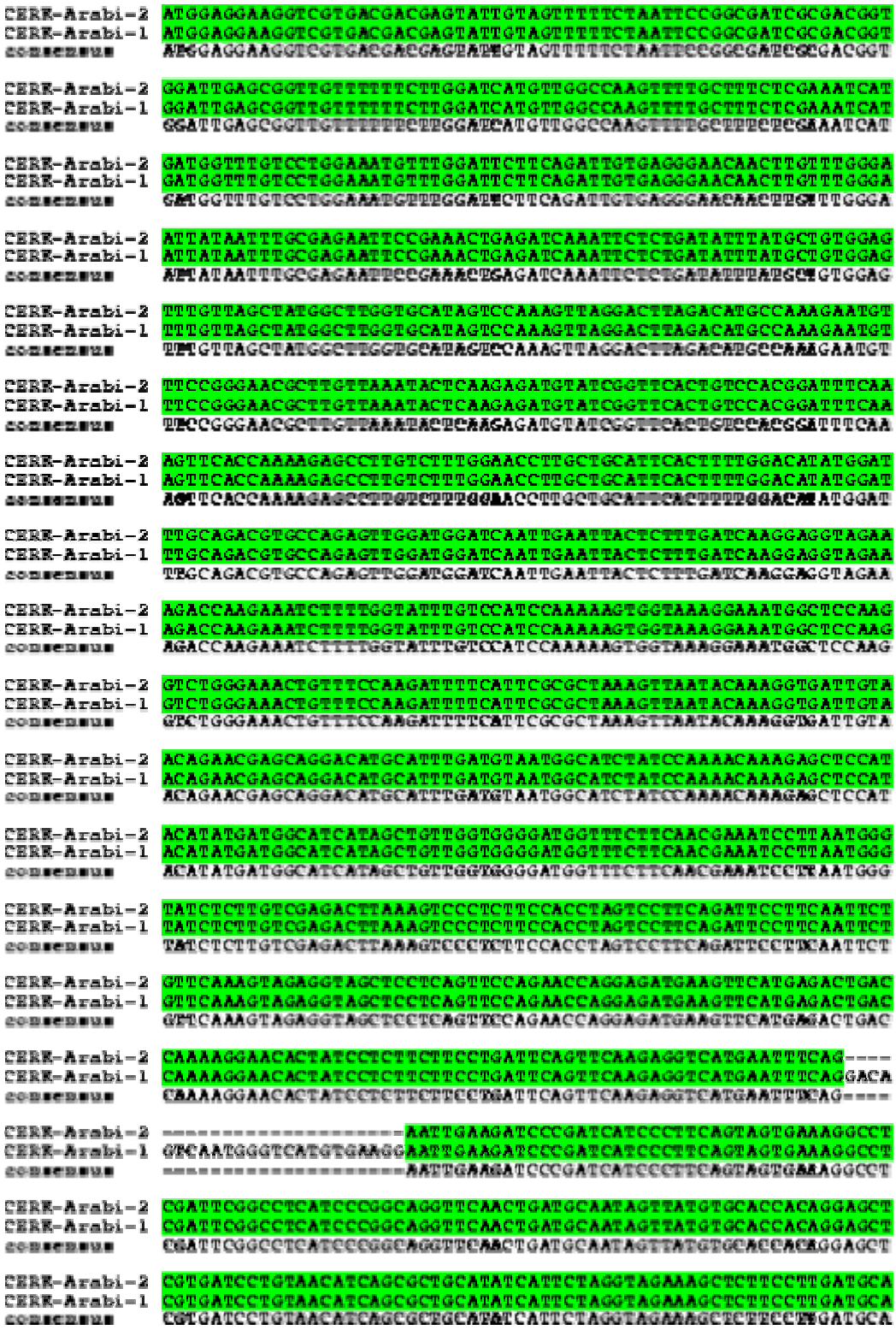


Figure 5.2. Nucleotides sequences alignments of the two transcripts of ceramide kinase in Arabidopsis

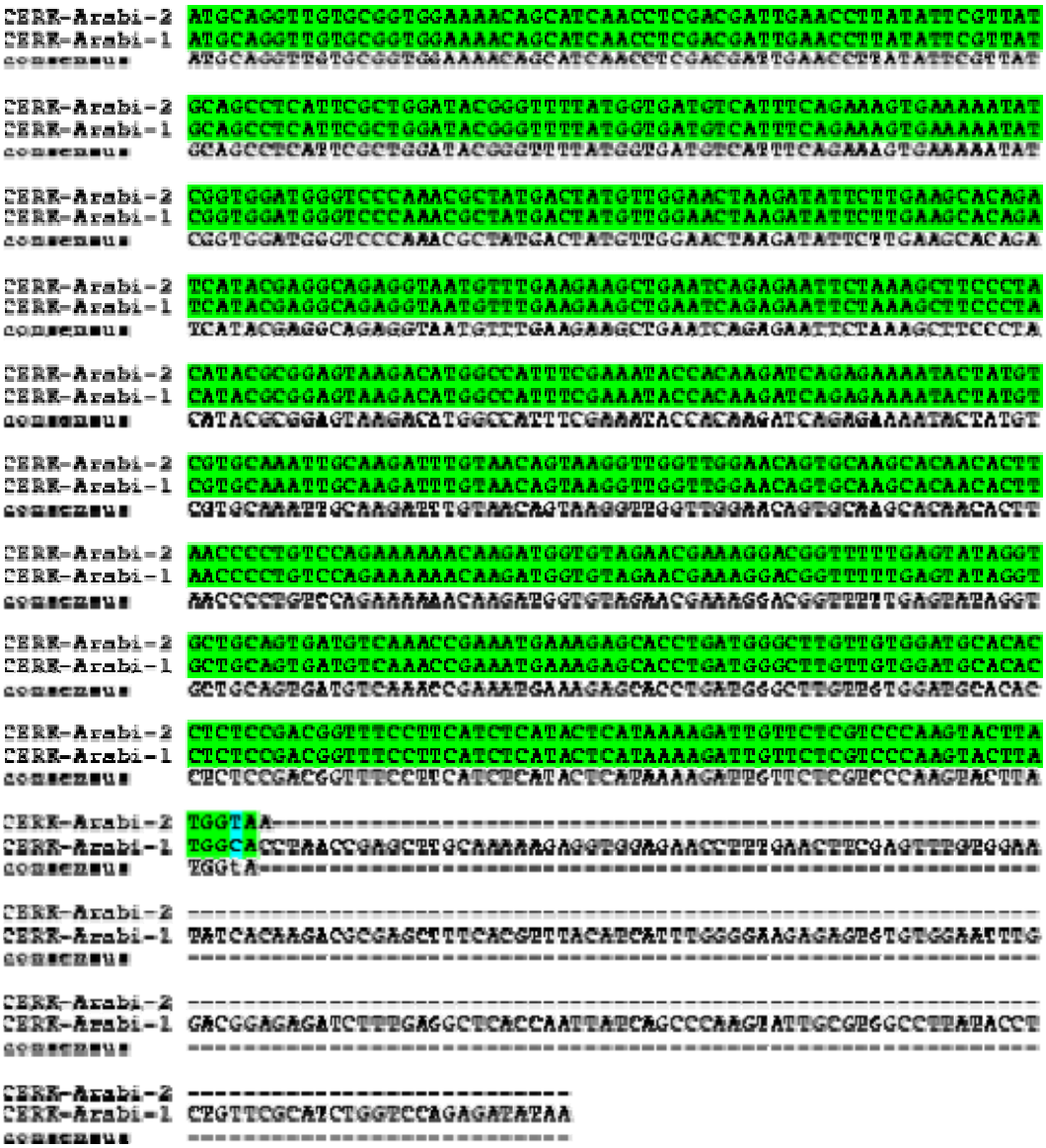


Figure 5.2. Nucleotides sequences alignments of the two transcripts of ceramide kinase in Arabidopsis (continued)

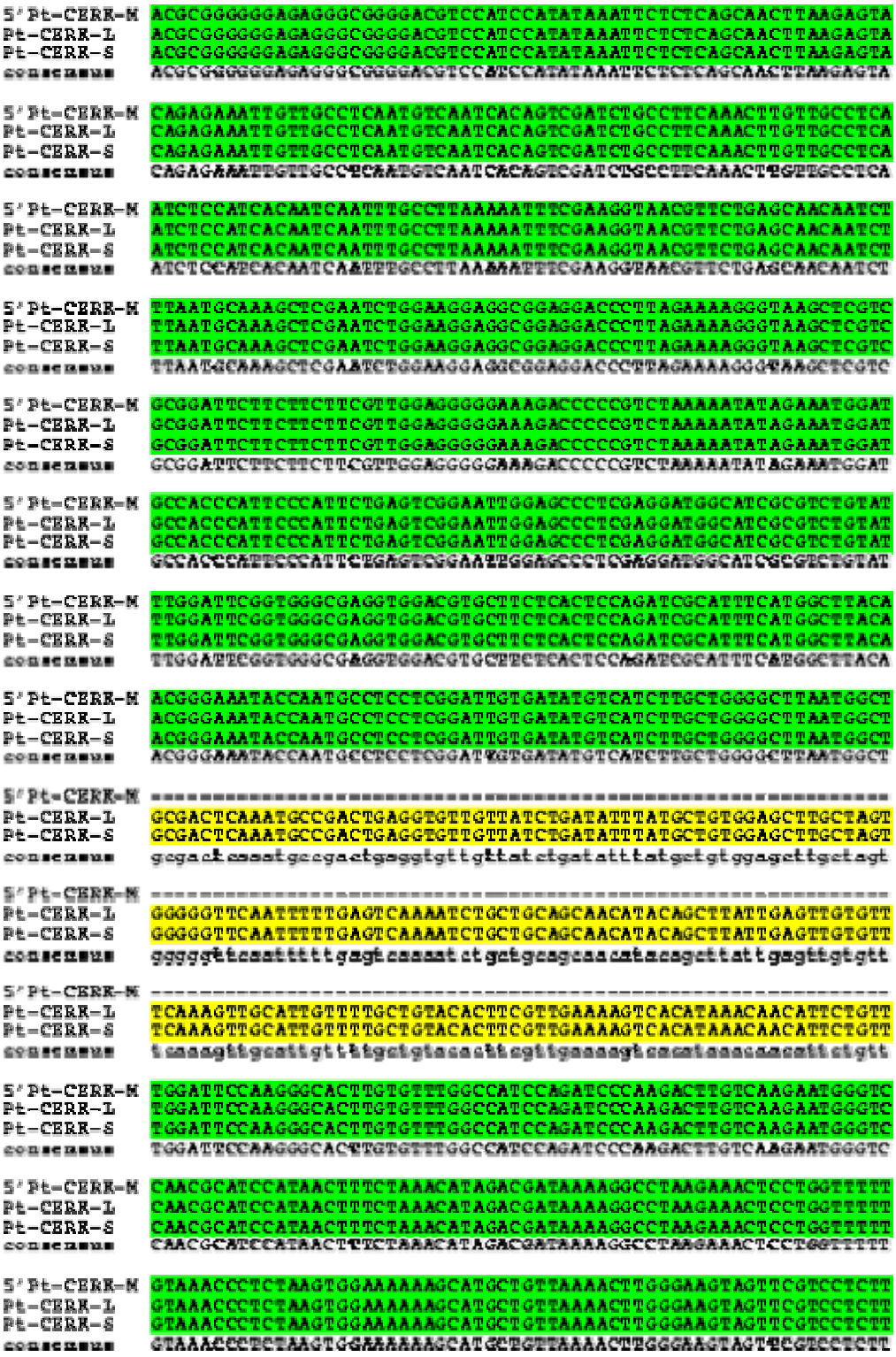


Figure 5.3. Nucleotides alignment results of the cloned full-length Pt-CERKs
The Pt- CERK-L, 5'RACE fragment of Pt-CERKm and Pt-CERK-S. Pt-CERK-S lost 486 bps nucleotides compared to the Pt-CERK-L. 180 nucleotides absent in the 5'RACE fragment of Pt-CERKM.

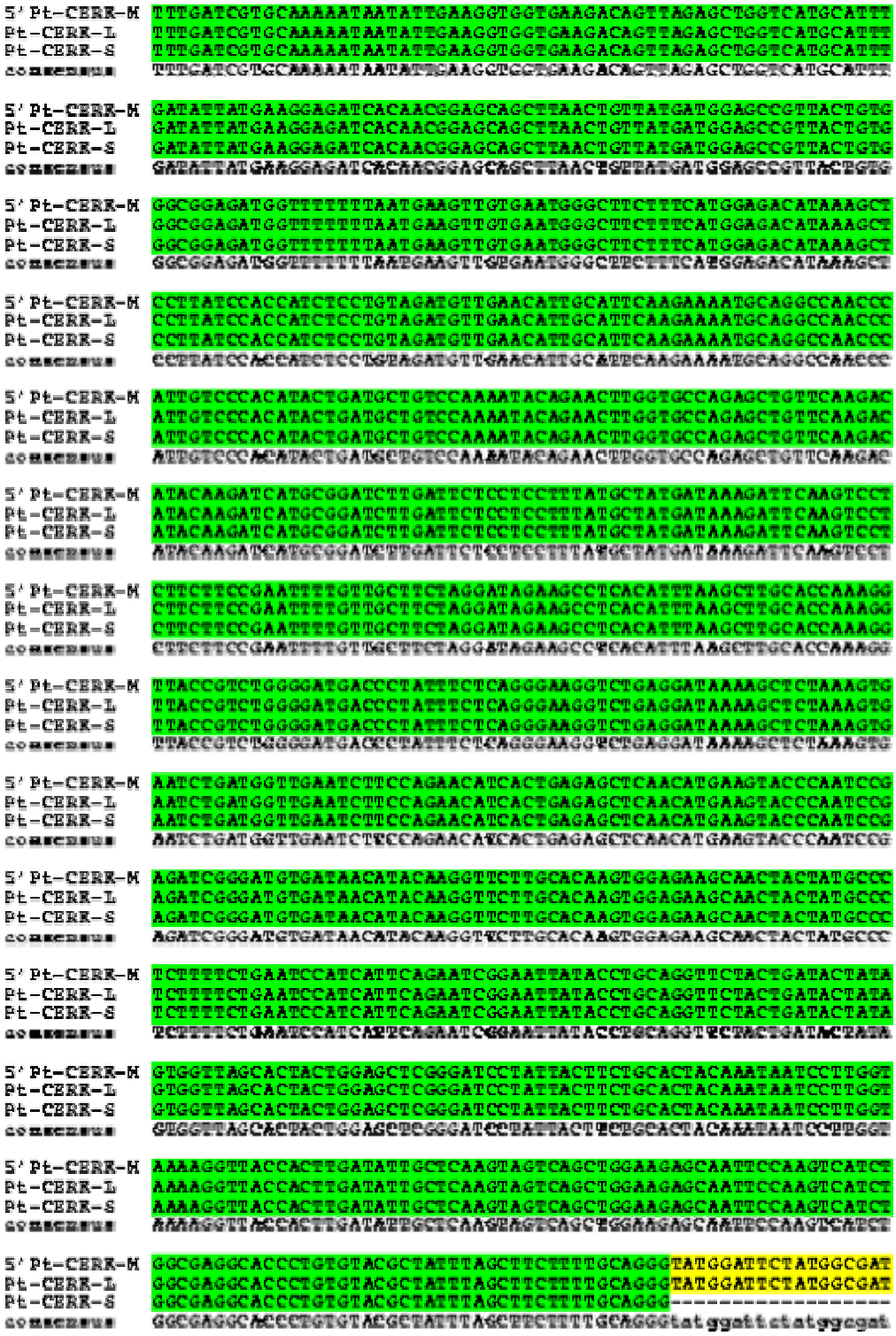


Figure 5.3. Nucleotides alignment results of the cloned full-length Pt-CERKs (continued)

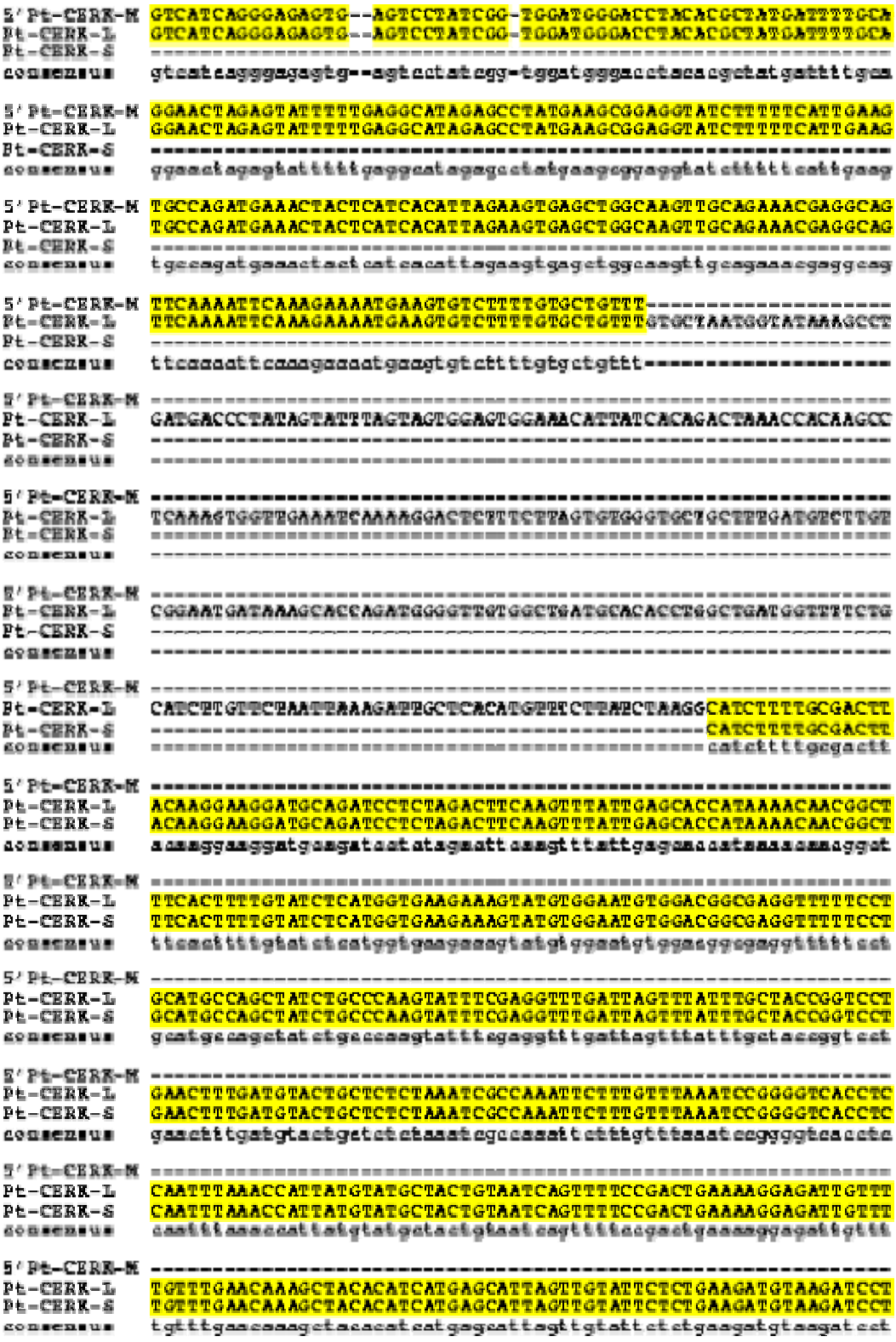


Figure 5.3. Nucleotides alignment results of the cloned full-length Pt-CERKs (continued)

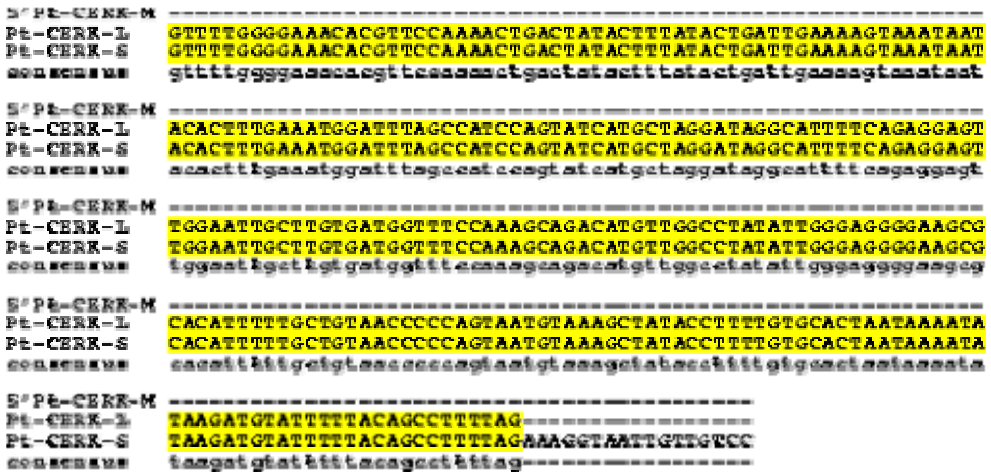


Figure 5.3. Nucleotides alignment results of the cloned full-length Pt-CERKs (continued)

5.3.2. Structural comparison and analysis among various versions of Pt-CERKs

SMART protein domain prediction was conducted for protein encoded by the cloned long version Pt-CERKL, short version Pt-CERKS, and 5'RACE fragment of the potential middle version Pt-CERKM. These results indicated that all three versions had the DAGK catalytic domain with a significant E value (Figure 5.1B, in gray and Figure 5.4, right in gray and light blue color, Figure 5.5, in gray). Both Pt-CERKL and Pt-CERKS have a Pleckstrin Homology Domain (PH) related region (Figure 5.1B, in green and Figure 5.4, right in green, Figure 5.5, in green). However, the 180 nucleotides loss leads to the PH domain absent in 5'RACE fragment encoding polypeptides (Figure 5.1B; Figure 5.3, the third column).

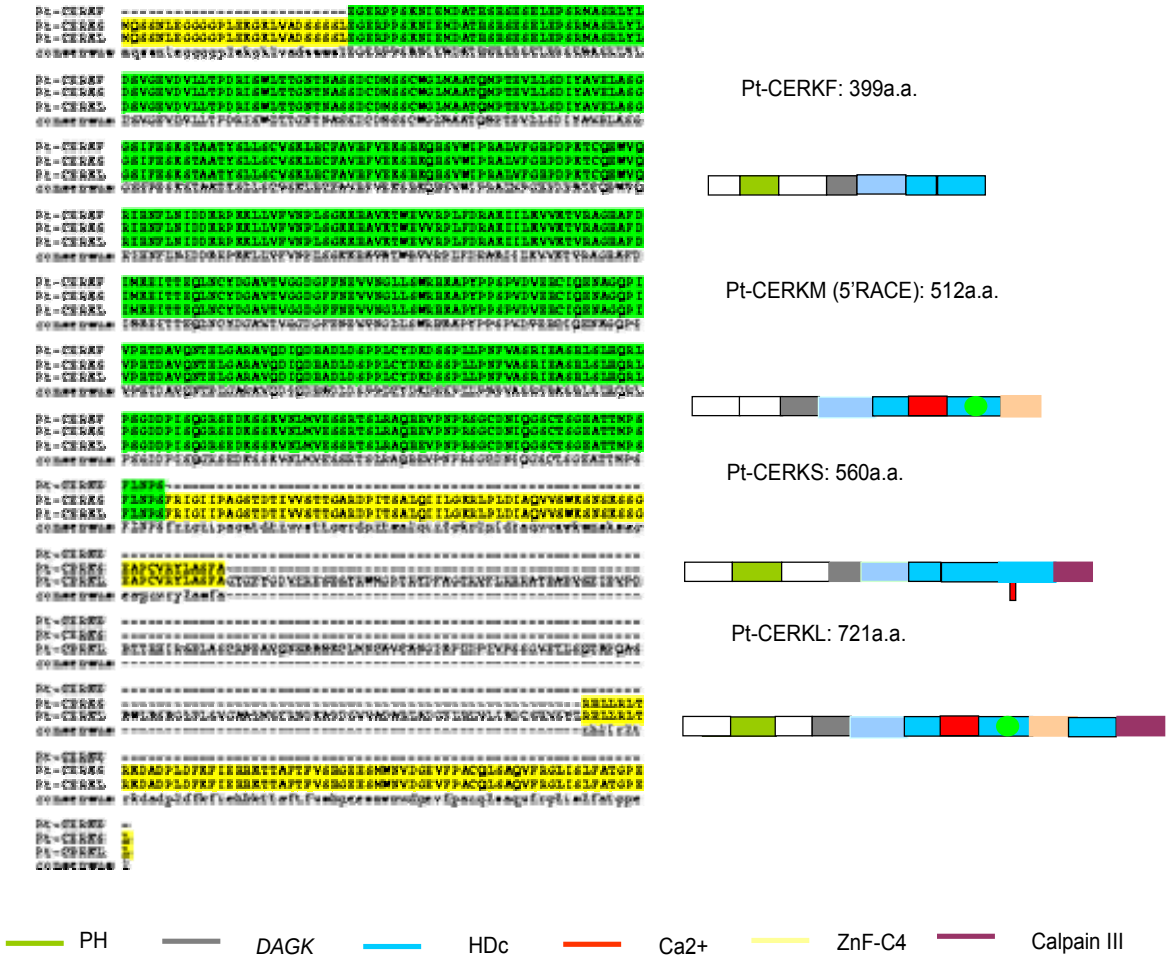
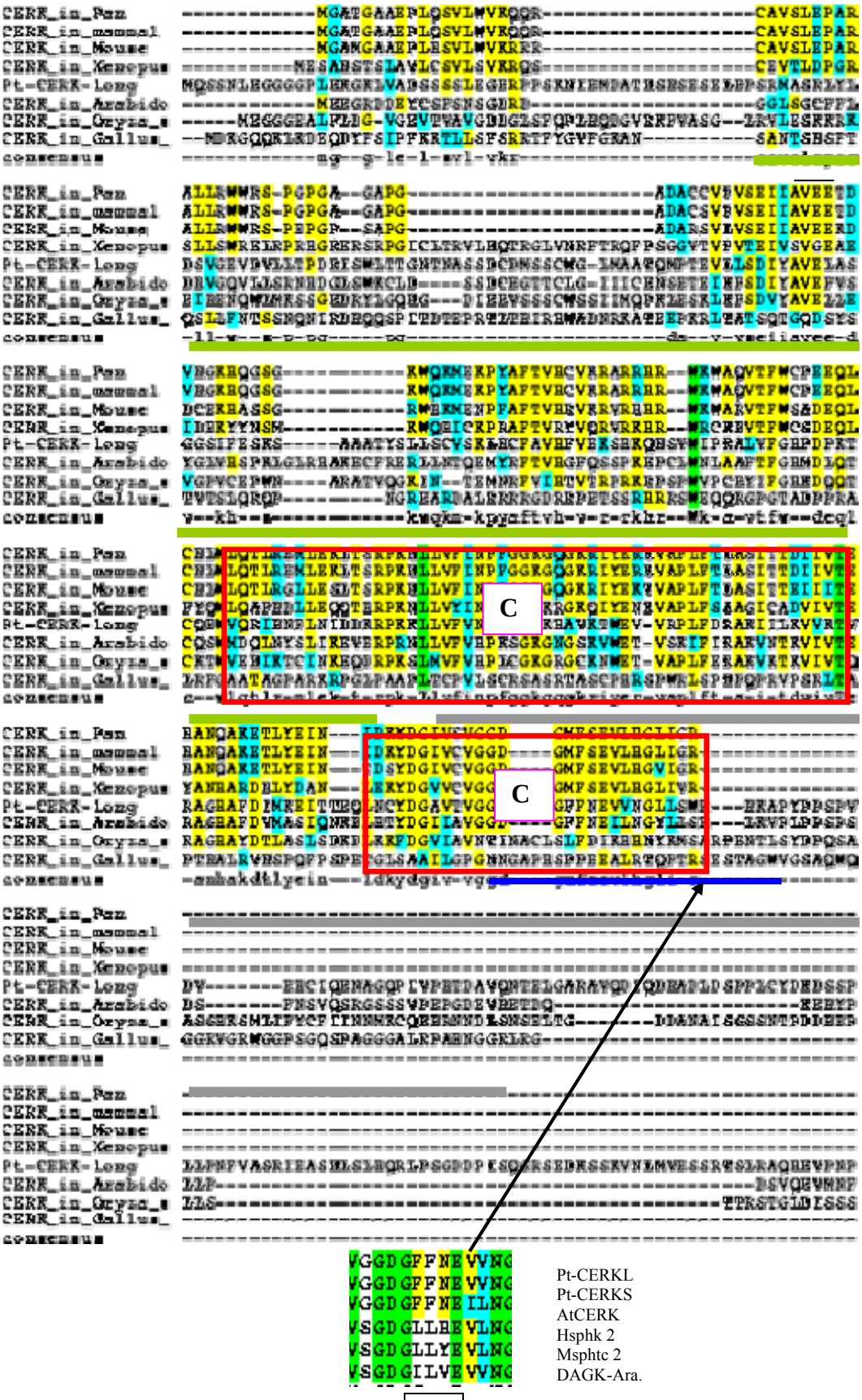


Figure 5.4. Alignment of the predicted amino acid sequence of the Pt-CERK clones used for protein expression and subsequent enzyme assay (left)
(See APPDEDIX 1 for clear view of the alignment result)
Diagram of the domain structures of these proteins (right). Pt-CERKs contain DAGK domain (dark gray and light blue), PH domain (green), HDc motif (light blue and blue) and ZnF_C4 (light pink) related regions. The conserved Glycine residue (green dot) and the calcium and calmodulin motif (in red)


C
C
Pt-CERKL
Pt-CERKS
AtCERK
Hsphk 2
Msphtc 2
DAGK-Ara.
A

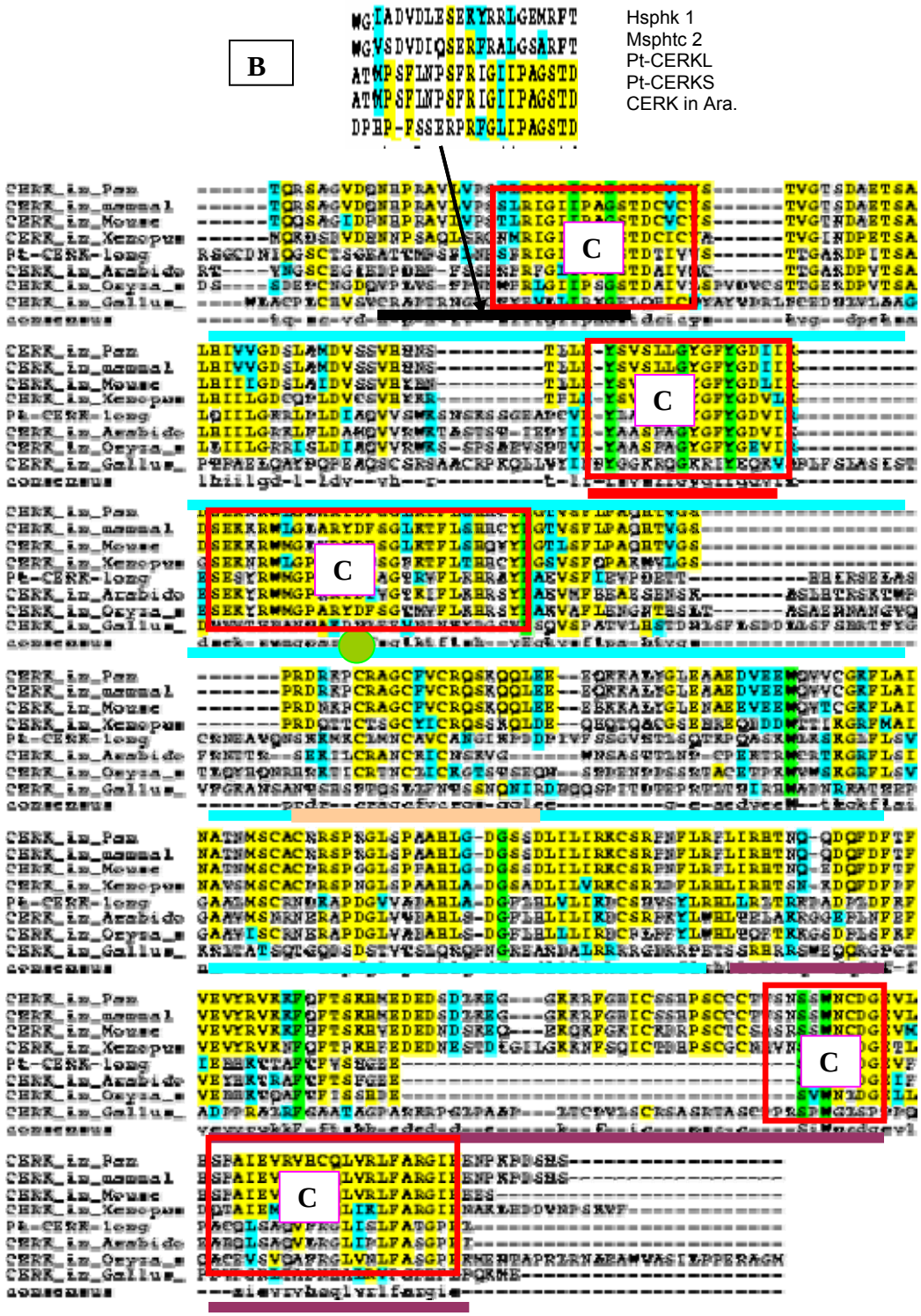


Figure 5.5. Alignment of different ceramide kinase sequence in various organisms
ClustalW alignment of the predicted amino acid sequences of ceramide kinase in Loblolly pine, Arabidopsis (AAQ62904), mammals (Q8TCTO), Pan (XP_001137835), Mouse (Q8K407), Rice(BAD25678), Xenopus laevis (NP_001086227) and Gallus gallus(NP_001026511). The consensus sequence represents amino acid that is conserved in five regions with transparent boxes. The PH domain's DGK catalytic domain, calcium/calmodulin domain, APT and substrate binding motifs and HDc domain

depicted by the SMART search tool are indicated by underlines and all located in the above five conserved domains. **A:** The conserved ATP binding motif, GXGXXXG, is highly conserved through DAGK in arabidosis to CERK in pine and Arabidopsis and then to Sphingosine kinase in human and mouse. **B:** The substrate binding motif in Sphingsine kinase of mammals is different to that of ceramide kinase in pine and Arabidopsis, while, both sequence of Sphingosine kinase 1 and Sphingosine kinase 2 have 64% identity and the identity of the both CERK in pine and Arabidopsis is 54%. Arrow points D is the 177 residue in Sphingosine kinase 1 in human.

The PH domain has been reported to have several different regulatory functions (Sugiura et al. 2002). For example, PH domain-containing proteins can bind to the β/γ subunit of heterotrimeric G-proteins, to phosphatidylinositol-4,5-bisphosphate, and to phosphorylated tyrosine residues (Gibson et al. 1994). Recently, it has been reported by Kim that the PH domain of *h*CERK was involved in plasma membrane attachment by binding to PI(4,5)P2; and as a result C1P is generated in the vicinity of the membrane (Kim et al. 2006). These results indicated the PH domain of ceramide kinase might not only be important for membrane anchoring but may also regulate CERK activity (Sugiura et al. 2002). Wattenberg et al suggested that although diacylglycerol kinase and sphingosine kinse were the members of the DAGK superfamily, they did not have an obvious transmembrane domain; both were presumed to have transient connection with the membrane that contained their substrates (Wattenberg et al. 2006). Like DAGK, the catalytic efficiency of ceramide kinase can be greatly improved by the phosphatidylserine (PA) and phosphatidic acid (PC) (Wattenberg et al. 2006). Further research has shown that the binding of DAGK or phospholipids to the PH domain of enzymes in DAGK family could affect the enzyme activity (Sugiura et al. 2002; Wattenberg et al. 2006).

Transmembrane prediction for Pt-CERK proteins by program TMPRED (http://www.ch.embnet.org/software/TMPRED_form.html) indicated that both the long version and short version were not transmembrane proteins (data not shown). So the only

PH domain in Pt-CERKs may have the same functions as that in DAGK and its homolog *h*CERK in human. Without a PH domain, the potential middle size protein will not bind to the membrane and should be in the cytosol phase. Its function or substrates might also be different from the function or substrates of Pt-CERKL and Pt-CERKS. Regarding the subcellular distribution of the kinase activity in mammals, contradictions exist (Bajjalieh et al. 1989; Sugiura et al. 2002). The ceramide kinases in mammals were previously reported to be membrane-associated (Bajjalieh et al. 1989; Kolesnick & Hemer 1990.). Upon overexpression of CERK in HEK293 cells, a membrane bound activity was found again (Gomez-Munoz 2004). But, Mitsutake et al (Mitsutake et al. 2004) recently showed that *h*CERK was mainly localized in the cytosol of RBL-2H3 cells but also present at the plasma membrane in a few cells (Mitsutake et al. 2004). Carre et al (Carre et al. 2004) reported a Golgi localization in Cos-1 and HUVEC cells, and a translocation of *h*CERK to the plasma membrane upon osmosis. The multiple versions of CERK that I have demonstrated in pine, and subsequently identified in other organisms show characteristics that could direct these proteins to different cellular compartments. Workers assigning various subcellular locations of ceramide kinase might be detecting different versions of CERK.

Research by Liang demonstrated that a mutant ceramide kinase (ACD5) in Arabidopsis showed enhanced disease symptoms and apoptotic like cell death during pathogen attack due to the change of glycine at residue 412 to arginine in this mutant ceramide kinase (Liang et al. 2003). This glycine residue was conserved in most ceramide kinases found in public databases (Liang et al. 2003). In this study, I show (Figure 5.4, right in green dot, Figure 5.5, green dot) that the conserved glycine is present in Pt-CERKL and the 5'RACE fragment of the potential middle size CERK in Loblolly

pine but absent in Pt-CERKS. This specific residue is conserved from *Gallus gallus* through Arabidopsis and rice then to human (Figure 5.5, right, green dot).

In addition, there are some particular domains within Pt-CERK proteins, such as the HDc domain, which are present in all cloned Pt-CERK proteins (Figure 6.4, in light blue and blue color, Figure 5.5, in blue underline). The HDc domain is always observed in a metal dependent phosphohydrolases superfamily (Aravind & Koonin 1998). It has been reported that all the highly conserved residues in this phosphohydrolases superfamily are histidines or aspartates. The proteins in this superfamily play vital roles in signal transduction and require divalent cations in their activities (Aravind & Koonin 1998). The HDc domain in Pt-CERKs (Pt-CERKL and Pt-CERKS) can be divided into two parts; the first part N-terminal is fused with the DAGK domain (Figure 5.4, right in light blue), and the second part (C-terminer), in which the highly conserved histidines and aspartates are accumulated for both Pt-CERK proteins, accounting for 11% in the second part of Pt-CERKL and 16% in the second part of Pt-CERKS; so the second part HDc domain is probably more like a phosphohydrolase (Figure 5.4, right in dark blue). *h*CERK (Q8TCT0) has a calcium/calmodulin binding motif, RYSVSLLGYGFYGE (residues 422 to 435), which is located just inside the HDc domain of *h*CERK (Sugiura et al. 2002). The highly conserved calcium/ calmodulin like motif 'RYLASFAGYGFYGD' is present in Pt-CERK proteins and also locates in this HDc domain, specifically in the conserved C4 domain (Figure 5.5, in red underline). This HDc motif is in residue 486 to 499 for the long version and in residue 426 to 439 for 5'RACE coding fragment (Figure 5.5, in red underline); however, more than half of this motif, AGYGFYGD, is lost in the short version (Figure 5.4, right, in red). Furthermore, there is only one residue difference

in this calcium/calmodulin domain between CERK in Arabidopsis and Pt-CERK-L. The identity of the calcium/calmodulin motif between *h*CERK and Pt-CERK is 71% as shown in Table 5.1.

Table 5.1. Comparison of ceramide kinases in various organisms
*: The principle CERK in each type of organism, of which the catalytic function, proved.

	Human	**Loblolly pine**	**Arabidopsis**	**Rice**
mRNA *mRNA/ Acc.No.* *Protein/Acc. No.*	1614/NM_022766 537*/NP_073603	2786 721*	1827/AY362552 608* /AAQ62904	1761/AJ307662 586/ CAC39069
mRNA/ Acc.No. *Protein/Acc. No*		2320 560	1602/AB023044 533/BAA97392	2103/AP006006 700*/BAD25678
mRNA/ Acc.No. *Protein/Acc. No.*	606/BC108712 201/AAI08713	1700 512		579/AP004772 192/ BAD25377
DAGK *	APT dependent enzyme	all three have	APT dependent enzyme	ATP dependent
Ca^{2+} binding (domain) *	RYSVSLLGYGFY GD (422-435) Ca^{2+} stimulates its activity	721,512 RYLASFAGYGF YGD	RYAASFAGYGFYGD 392) Ca^{2+} enhance CERK activity	RYAASFAGYGF YGE (458-472)
PH domain*	γ/β subunits of heterotrimeric G-protein, PIP2 and phosphorylated tyrosine. CERK target plasma membrane	721 560	608*	700*
Mutant CERK*			High activity on synthetic C6 and C8 ceramides, 10-20 fold less activity on natural ceramide and didydrceramide.	
Catalytic function*	Response to a wide range of stress and promotes phagolysome	Both long and short versions have the catalytic functions	Mutant CERK enhanced disease symptoms during pathogen attack cell death dependent on stress SA. and ethylene	
C1P*	Fusogenic: Regulate secretion of Neurotransmitters Mitogenic: stimulate DNA synthesis Pathogenic: Arachidonic acid synthesis			

This motif is highly conserved through rice to human. Even rice CERK has 71% identity with *h*CERK in this calcium/calmodulin motif (Table 5.1). This conserved domain showed an YXXXXXGXXXYGD pattern from *Gallus gallus* to mammals (Figure 5.5, in red uderline). Obviously, the absence of eight highly conserved residues implies that Pt-CERKS has lost the function specific for this region. Previous research by Pettus has demonstrated that the translocation of cPLA2 is induced by ceramide 1-phosphate through the Ca^{2+}-dependent lipid binding domain (CaLB/C2) to Golgi and peri-nuclear membranes (Pettust & Bielawska 2004). Ceramide 1- phosphate as the product of ceramide kinase, works together with ceramide kinase. Probably, the calcium/calmodulin domain in Pt-CERKs has a similar function to bind its substrate and to bring the enzyme to a membrane system inside cell or by binding to a target protein to relocate its product, C1P. In another report by Bajjalieh indicated that CERK activity could be stimulated by Ca^{2+} (Bajjalieh et al. 1989). Sugiura et al (Sugiura et al. 2002) also reported a Ca^{2+} dependence for the recombinant CERK which could reach a maximum at ~0.5 mM for free Ca^{2+}. Research by Liang et al in 2003 indicated that the catalytic reaction of recombinant ACD5 CERK (expressed in *E.coli*) was stimulated by Ca^{2+} (Liang et al. 2003). However, the research by van Overloop et al (van Overloop el al. 2006) showed that *h*CERK displayed high kinase activity in the absence of Ca^{2+} but in the presence of Mg^{2+} (van Overloop el al. 2006). Obviously, for the catalytic functions of CERKs in various organisms, a contradiction exists. The presence of multiple forms of CERKs in Loblolly pine, one of which, as demonstrated earlier, lacks a Ca^{2+} binding site, might resolve the apparent contradiction in the results from different laboratories. The

versions that have the Ca^{2+} site would be expected to sense to Ca^{2+} stimulation, while for those lacking the specific Ca^{2+} binding motif but with the catalytic domain probably will not depend on the Ca^{2+} ions.

Another significant motif, ZnF_C4, which is C4 Zinc finger in nuclear hormone receptors (Gronemeyer & Laudet 1995) (Figure 5.4 , in light pink, Figure 5.5, in light pink) exists in Pt-CERKL and the 5'RACE coding fragment but is absent in Pt-CERKS. The loss of ZnF-C4 in the short version probably makes it insensitive to certain plant hormones. Zinc finger motif has been found in several human hormone receptors and a particular form of this motif is found in the steroid receptor (Lewin 2004). Steroid or nuclear hormone receptors constitute an important superfamily of transcription regulators that are involved in widely diverse physiological functions, including embryonic development, cell differentiation and homeostasis (Lewin 2004). Based on the prediction by *Wolf Psort* (http://wolfpsort.org/), both Pt-CERKL and Pt-CERKS are likely to be nucleus and ER localized. So, Pt-CERK proteins might locate to the ER and could be close to the nuclear envelope. The Pt-CERKL might not only work as a lipid kinase but also as a translocation protein and a receptor for certain types of hormone. In addition to the difference in ZnF-C4 domain, the calcium/calmodulin and the Glycine residue, another significant difference between Pt-CERKL and Pt-CERKS proteins is the region of unknown function; the calpain III domain. In Pt-CERKL, this unknown function domain is a neighbor to the ZnF-C4 region, while the calpain III domain in Pt-CERKS is connected to the HDc region (Figure 5.4, in purple, Figure 5.5, in purple underline) and calpain III in both proteins is mainly located in the conserved C5 region mentioned in the following paragraph. The calpain III domain in both proteins contains about 11-16%

histidine and aspartates. High histidine and aspartate are characteristics of phosphohydrolases. Probably, the Pt-CERK proteins are bi-functional protein of kinase/phosphohydrolase, which have been reported to exist in prokaryotic organisms and archae under certain signal controlling (Kennells 2003).

When aligning amino acid sequences of the ceramide kinases in Loblolly pine (Pt-CERKL), Arabidopsis, rice, chicker (*Gallus gallus),* chimpanzee (*Pan troglodytes)* and human, it is observed that ceramide kinase in Loblolly pine also contains five conserved domains (C1-C5) previously identified in the mammal sphingosine kinases (Liu et al. 2002; Sugiura et al. 2002) (Figure 5.5, transparent boxes). These conserved domains are likely involved in enzymatic catalysis and/ or the selection of specific substrates (Sugiura 2002). Those five domains including the ATP binding motif, the substrate binding domain, DAGK, Calcium/calmodulin, HDc, ZnF-C4 domains, and glycine residue, are conserved from Arabidopsis, rice, pine and all the way to human (Figure 5.5, boxes, HDc in blue underline, ATP binding in blue, DAGK in gray underline, substrate binding in black, calcium/calmodulin in red line, ZnF-C4 in light pink, glycine residue in green). There are slight variations in the five conserved domains among different organisms. These results might indicate the difference of their catalyzed substrates, and sensitivity to various exterior stimuli from their diverse living environments. As the member of the super-lipid kinase family, ceramide kinase shares many characters with sphingosine kinase and diacylglycerol kinase (Wattenberg et al. 2006). An identification of a DAGK mutation in Drosophila in the research of Pitson et al (Pitson et al. 2000) indicated that the ATP binding site had been identified as the GXGXXXG motif within the putative catalytic pocket in this super lipid kinase family (Pitson et al. 2000). This result is further

confirmed by other studies in which the second glycine in this motif was mutated to an aspartate or alanine, resulting in loss of the DAGK catalytical activities would lost (Topham & Prescott 1999). By aligning the Pt-CERKL protein sequence with the Arabidopsis CERK, Arabidopsis DAGK and the sphingosine kinase 2 in human and mouse, it is found this GXGXXXG-like region is conserved in all three kinds of lipid kinases (Figure 5.5, blue line and extra box A). In Loblolly pine this motif is in residues 260 to 271, which has about 90% sequence identity with ceramide kinase in Arabidopsis, 61% sequence identity with sphingosine kinase 2 in mammals, and 54% sequence identity with DAGK in Arabidopsis (Figure 5.5, extra box A). This motif might be the ATP binding region for ceramide kinase. Research by Yokota et al suggested the sphingosine kinase C4 region was involved in sphingosine binding due to the fact that mutation of aspartate 177 within the C4 region increases the Km of sphingosine kinase 1 for sphingosine (Yokota et al. 2004). When aligning protein sequences (C3 region) for Pt-CERK proteins, CERK in Arabidopsis, spingosine kinase 1 and sphingosine kinase 2 (C4 region) in human and the DAGK in Arabidopsis, the results are as expected: these specific sequences are different between sphingosine kinase group and ceramide kinase group because these two groups catalyze different categories of substrate (Figure 5.5, extra box B). However, in the same group, sphingosine kinase 1 and sphingosine kinase 2 have 64% sequence identity in the substrate binding region. The substrate binding region in ceramide kinase in Loblolly pine and Arabidopsis has 54% sequence identity (Figure 5.5, extra box B). So, it is most likely that sequence ‘PSFLNPSFRIGIIPAGSTC’ is a substrate binding related region in ceramide kinase in Loblolly pine.

5.3.3. The putative CERK proteins in Loblolly pine have CERK catalytic function similar to their homologs in mammals and Arabidopsis

The CERK long version (Pt-CERKL) and CERK short version (Pt-CERKS) ORF cDNAs were obtained by PCR amplification on the plasmid pGEM T Easy in which I had initially cloned the cDNAs using primers (with restriction enzymes NheI and EcoRI sequences) Pl-F-CERK (5'-CTAGCTAGCTTCGAAGGTAACGTTCTGAGCAACAA3');Pl-R-CERK (5'- CCGGAATTCTTGGAGGTGACCCCGGATTTAAACA-3'), respectively. To investigate the effect of the last unknown function domain in the short version, Pt-CERKS, a short fragment of Pt-CERKF was created by PCR amplification on the Pt-CERKS stored plasmid pGEM T Easy vector with primers Pf-F-CERK (5'-CTAGCTAGCGAGGGGGAAAGACCCCCGTCTAA-3') and Pf-R-CERK(5'-CCGGAATTCTGATGGATTCAGGAAAGGAGGGCATAG-3') (Figure 5.4), which has the same pair of restriction enzymes. The sequences of all Pt-CERK cDNAs were checked by re-sequencing after cloning into expression vectors, pET28a+ (BD Clontech). The recombinant plasmid was transformed into *E.coli* strain BL21 for large-scale expression. 5 ml TALON® Single Step Column Purification were used to obtain pure Pt-CERK proteins (data not shown here).

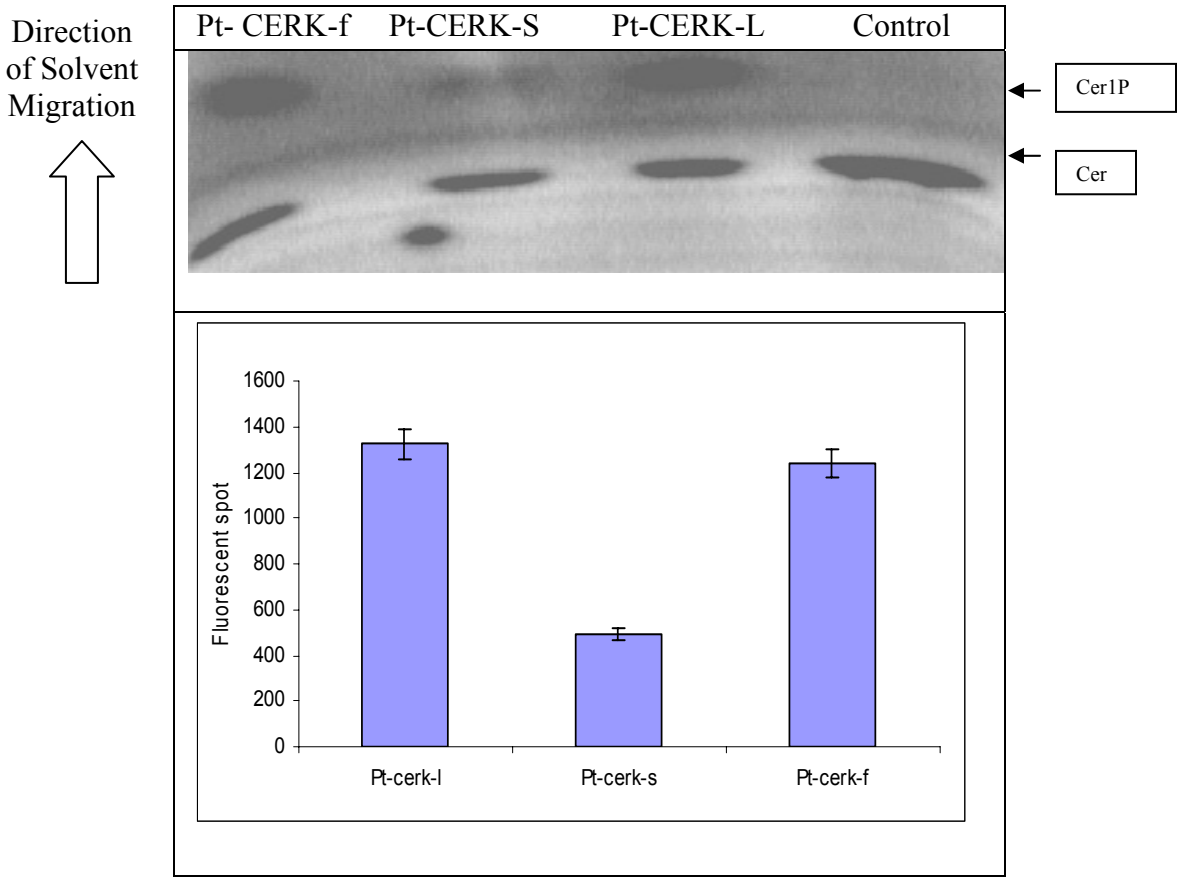


Figure 5.6A. Cloned two Pt-CERK enzymes with catalytic function to convert ceramide to ceramide 1 phosphate

A: The long version, Pt-CERKL and short version Pt-CERKS and the truncated fragment, Pt-CERKF all have the catalytic functions, but the Pt-CERKS has lower catalytic efficiency, because 30 times more Pt-CERKS protein had been added in reaction.

In vitro Assay of CERK functions using recombinant Pt-CERK proteins

Recombinant CERK proteins were assayed by incubating with a fluorescent cermide (NBD-Cer6) substrate in buffer containing ATP, Mg^{2+} and Ca^{2+} (See APPENDIX 1: Materials and Methods). The products were separated on a silica gel TLC plate using chloroform: methanol: water (25:20:1.1) as the solvent system. In Figure 5.6A, the presence of a slower migrated ceramide and a faster moving Cer1P indicate that the pine proteins can phosphorylate ceramide in vitro, thus the cloned Loblolly pine cDNAs

encode functional CERK enzymes. The quantity of products is presented graphically by columns (Figure 5.6 A, B, C), while the time course of enzyme activity is given for both Pt-CERKS and Pt-CERKL (Figure 5.6B).

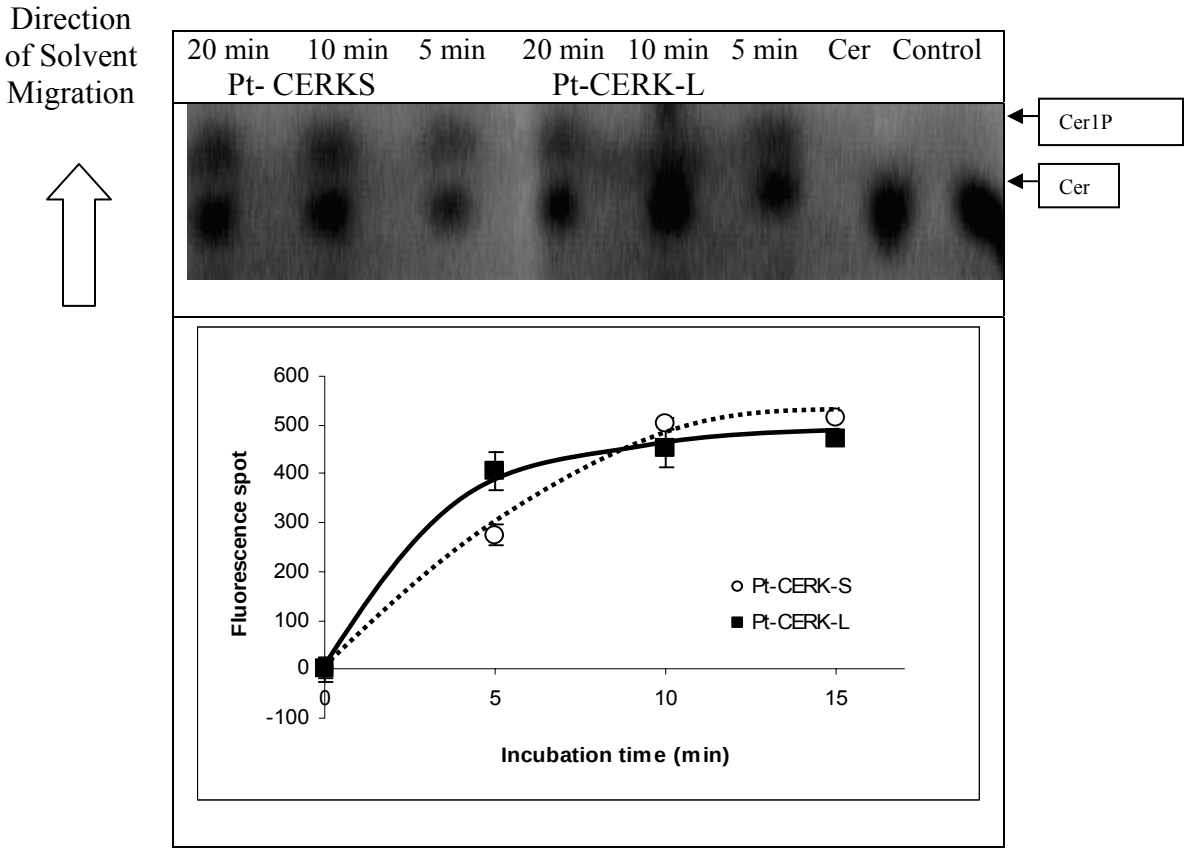


Figure 5.6B. Cloned two Pt-CERK enzymes have catalytic function to convert ceramide to ceramide 1 phosphate

Long version Pt-CERKL reaches its max productivities 5 minutes after the reaction started, while the short version reaches it max amount at 10 minutes.

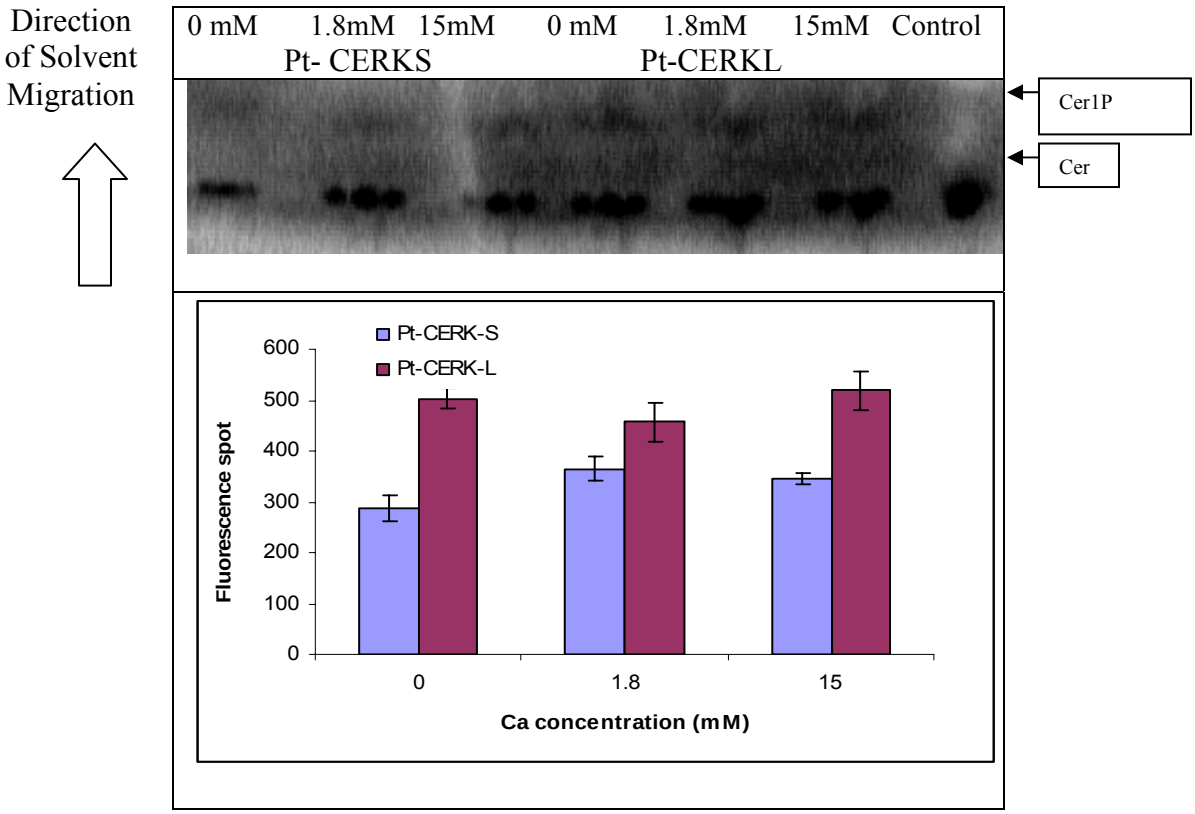


Figure 5.6C. Cloned two Pt-CERK enzymes have catalytic function to convert ceramide to ceramide 1 phosphate
Both versions of Pt-CERK proteins are not depended on Ca^{2+}, because Ca^{2+} concentration changed from 0 mM to 15 mM did not lead a difference in both of them.

Pt-CERK enzyme assay results demonstrated that the putative long and short version proteins had the functions to catalyze phosphorylation of ceramide to form ceramide -1- phosphates (Figure 5.6 A).

To investigate whether the calcium ions have an effect on the catalytic reaction of pine ceramide kinase, enzyme assays for the two enzymes (Pt-CERKL, Pt-CERKS) were conducted with various Ca^{2+} concentration buffers (0 mM, 1.8 mM and 15 mM Ca^{2+}). My reactions were sampled after 15 minutes incubation and any initial differences in enzyme activities have not been observed for both long and short version ceramide kinase in

Loblolly pine; all p value are larger than 0.05 (Figure 5.6 C). All reactions at three different Ca^{2+} concentrations produced almost the same amount of C1P. In this assay the calcium/calmodulin domain in Pt-CERK enzymes most likely does not have direct effect on their kinase function. However, at 15 minutes both Pt-CERKS and Pt-CERKL enzymes have reached a plateau (Figure 5.6B). Any effect of Ca^{2+} on the initial activities of the enzymes would not have been in this assay. However, previous assays with Arabidopsis-extracts were conducted for 30 minutes and 100mM Ca^{2+} was used to stimulated activity. By contrast, Mitsutake et al (Mitsutake et al. 2004) conducted cell assay and regulated internal Ca^{2+} levels using overexpression.These results are different from the previously reported data by Susumu et al (Susumu et al. 2005) and Linag et al (Liang et al. 2003) that calmodulin was involved in the Ca^{2+} dependent activation of ceramide kinase as a calcium sensor; and C1P was demonstrated as the product of a Ca^{2+} stimulated kinase that was purified from brain synaptic vesicles (Bajjalieh et al. 1989; Sumusu et al. 2005). However, the enzyme assay results from Pt-CERK enzymes agreed with the research by van Overloop et al that the bacterially expressed *h*CERK was less dependent on Ca^{2+} ions (van Overloop et al. 2006). Further, Calcium/calmodulin might be needed for substrate accumulation or transport CERK to substrate abundant space within cells by sensing the cellular Ca^{2+}, which do not need by the in vitro reaction.

To estimate the dynamic pattern of pine ceramide kinase through time, enzyme assays of the two versions (Pt-CERKL and Pt-CERKS) were incubated at 37°C for different time periods (5, 10, and 20 minutes). The experiment results (Figure 5.6 B) demonstrated that Pt-CERKL reached its maximum activities in 5 minutes after the reaction started; the Pt-CERKS activity reached its maximum at around 10 minutes but

its product, C1P, had a slight decrease after 20 minutes incubation (Figure 5.6, B). Statistical analysis did not show a significant difference for this decrease with the p value 0.15338. Hence, there were significant difference in the amount of the products between 5 minutes and 10 minutes incubation and a significant difference between 5 minutes and 20 minutes incubation with p vale 0.00031 and 0.00011, respectively in short version ceramide kinase (Pt-CERKS) assay.

In this enzyme assay, ceramide substrates were directly added to 5X reaction buffer and mixed with the purified Pt-CERK proteins without providing the substrate micelles. The results indicated that micelles were not necessary components for Pt-CERK proteins (Figure 5.6 A-C).

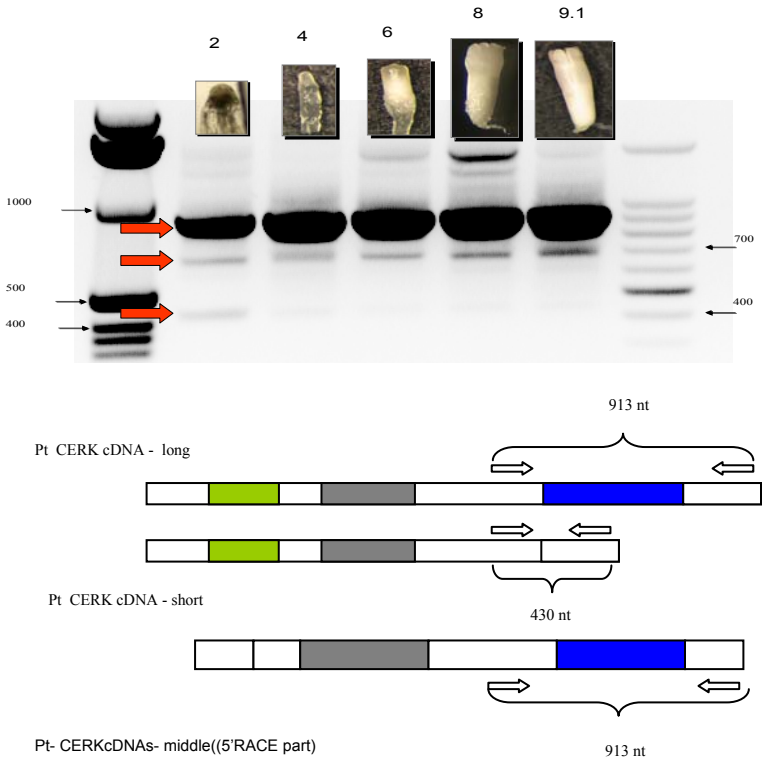


Figure 5.7. The expression pattern of the long and short version ceramide kinase genes during embryo development through the early stage, middle stage to the late stage

Pt-CERK expression patterns during embryo development

To study the expression pattern of Pt-CERK genes, two pairs of specific primers were designed and used in RT-PCR reactions. The first pair of primers was: Forward: GAAGTACCCAATCCGAGATCGGGATGTGATAA and Reverse: TCGACGAAAAACCTCGCCGTCCAC (see Figure 6.1A for confirming the existence of short version); and the second pair was: Forward: 5'-CTAGCTAGCTTCGAAGGTAACGTTCTGAGCAACAA3': and Reverse: 5'-CCGGAATTCTTGGAGGTGACCCCGGATTTAAACA-3'. The expected results for the longer fragment (913 bps) in the long transcript and the short version (431 bps) in short transcript were obtained and shown in Figure 5.7.

The results in Figure 5.7 clearly indicated that the Pt-CERKL mRNAs is expressed almost in the same amount during embryo development of Loblolly pine through the early, middle to the late stage in 2004 embryos (Using primer : Forward: GAAGTACCCAATCCGAGATCGGGATGTGATAA and Reverse: TCGACGAAAAACCTCGCCGTCCAC). The potential Pt-CERKM should has the same size here as Pt-CERKL; the Pt-CERKS mRNAs is only observed in the early stages (Stage 2) in developing embryos (Figure 6.7). The above Semi-quantitative RT-PCR started with same amount of total RNA (210 ng). Although we still used the same amount of total RNA (210 ng) to do Semi-quantitative PCR using embryos collected from 2005 (using primers: Forward: 5'-CTAGCTAGCTTCGAAGGTAACGTTCTGAGCAACAA3': and Reverse: 5'- CCGGAATTCTTGGAGGTGACCCCGGATTTAAACA-3'), the PCR results showed that long version expression was almost the same through stage 1 to stage 9.1, except the stage 5, which was less than the other stages (Figure 6.8, right). However,

short version showed different expression pattern compared to the embryos from 2004 (Figure 5.7, Figure 5.8); it did not only showed up in early stage , such as, stage 3, 4, but also appeared in middle stage 6, and even in late stage 8 (Figure 5.8). The band in the middle of the Pt-CERKL and Pt-CERKS is probably the Pt-CERKM. We failed in cloning the full size Pt-CERKM because of its less expression. In somatic embryos, long version expressed and displayed in a gradually increasing pattern, while the Pt-CERKS and Pt-CERKM did not show up through all the stages (Figure 5.8). Obviously, Pt-CERK expression patterns are embryo development stage related and probably also be influenced by some other environment factors, such as plant hormones (ethylene, the phenolic salicylic acid or other steroids**),** the individual mother trees, pathogen, stress etc. The experiment results also suggested that the CERK short version and the potential middle version expression were not actively expressed, their expression is closely incorporated to the embryo development phases and their on or off might be very sensitively controlled by the exteriors stimulus. Refer to the Pt-CERK expression in somatic embryogenesis, only the long version actively expressed and in a gradually increase pattern, which is significant different from the Pt-CERK expression in zygotic embryogenesis. In the semi-quantity PCRs, identical amounts (210ng) of total RNA were used for cDNA synthesis. Conventional controls such as monitoring the level of actin mRNA have proven inexact since the actin family is large in pine. Recently primers have been designed that are successful for pine actin mRNA, (Cairney personal communication) however these and the necessary protocols were not available to me at the time. The cDNA created and used in these experiments has been used by others in the laboratory and consistent, verifiable results have been obtained. For this reason, while the

lack of a control transcript limits interpretation, there is good reason to believe that the general trends that were observed are accurate.

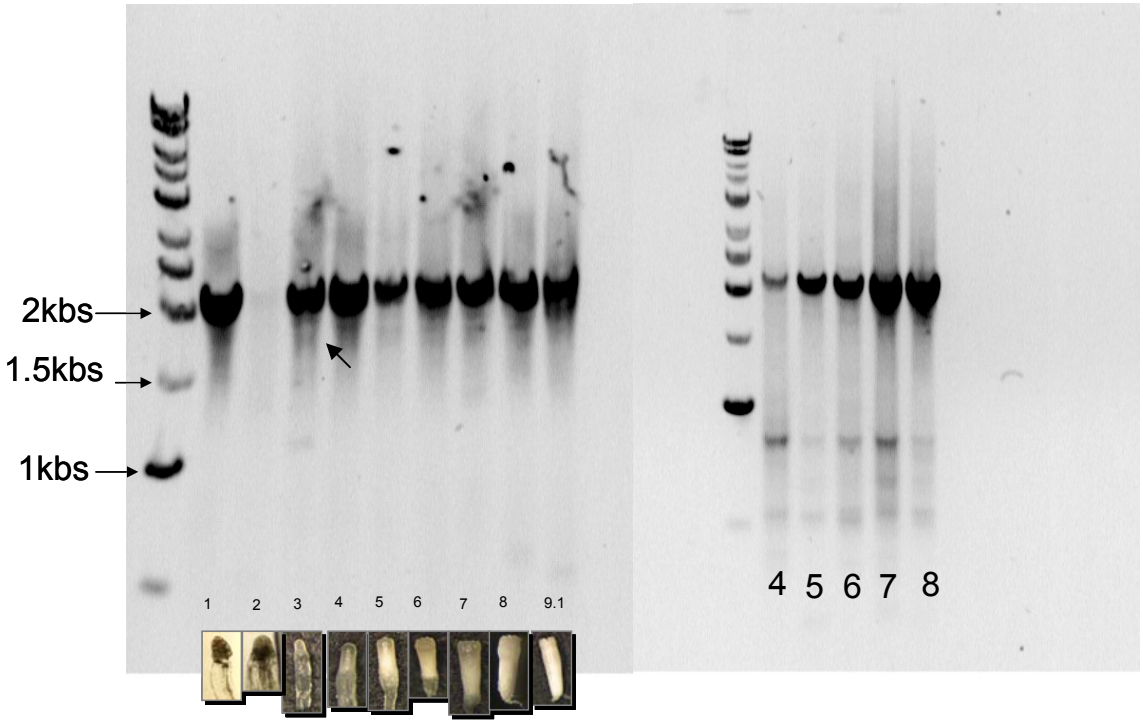


Figure 5.8. The expression pattern of CERK long version and short version in zygotic and somatic embryos of Loblolly pine
CERKs in zygotic embryos from stage 1 to stage 9.1 (left). CERKs in somatic embryos of LP from stage 3-4 to stage 8

It is known that somatic embryogenesis is a process that embryos develop under abnormal situation. Research by Greenberg indicated that acd5 mutant (The Glycine changed to Arginine) plants exhibited cell death late in development, which was largely dependent on the stress and defense signaling pathways controlled by the hormone ethylene and the phenolic salicylic acid (Greenberg et al. 2000).

Ethylene is a gas hormone, which can be induced by any type of wounding, such as flooding, chilling, disease, and temperature or drought stress. Ethylene functions by

binding to its receptor in a transmembrane domain through a transition metal cofactor, copper or zinc (Zaiz & Seiger 2002). The ethylene receptors, which are encoded by a family of genes, are plasma membrane or ER location. These receptors work similar to bacterial two-component histidine kinase. A serine/threonine protein kinase is also involved in ethylene signaling (Zaiz & Seiger 2002). However, currently no reports show any relationship among ethylene, ethylene receptor and ceramide kinase.

5.3.4. Evolutionary position of Pt-CERKs

Phylogenetic analysis shows that ceramide kinase in Loblolly pine is in a distinct class of lipid kinases that are distinguishable from sphingosine kinases and diacylglycerol kinase (Figure 5.9). Based on previously reported research, the DAGK domain was widely observed throughout evolution in sphingosine kinases, diacylglycerol kinases, and ceramide kinases (Nava et al. 2000). Specifically the human DAGK only phosphorylated diacylglycerol and not ceramide and sphingosine, whereas human sphingosine kinase only phosphorylated sphingosine (Nava et al. 2000). An exception is bacterial DGK, which phosphorylated both diacylglycerol and ceramide (Sugiura et al. 2002). Obviously, as with previous results (Sugiura et al. 2002), my phylogenetic analysis showed that after yeast, sphingosine kinase and ceramide kinase diverged into two large groups while sphingosine kinase was more closely related to DAGK than to ceramide kinase within the lipid kinase superfamily. Within the ceramide kinase group, animal and plants separate into two subgroups (Figure 5.9).

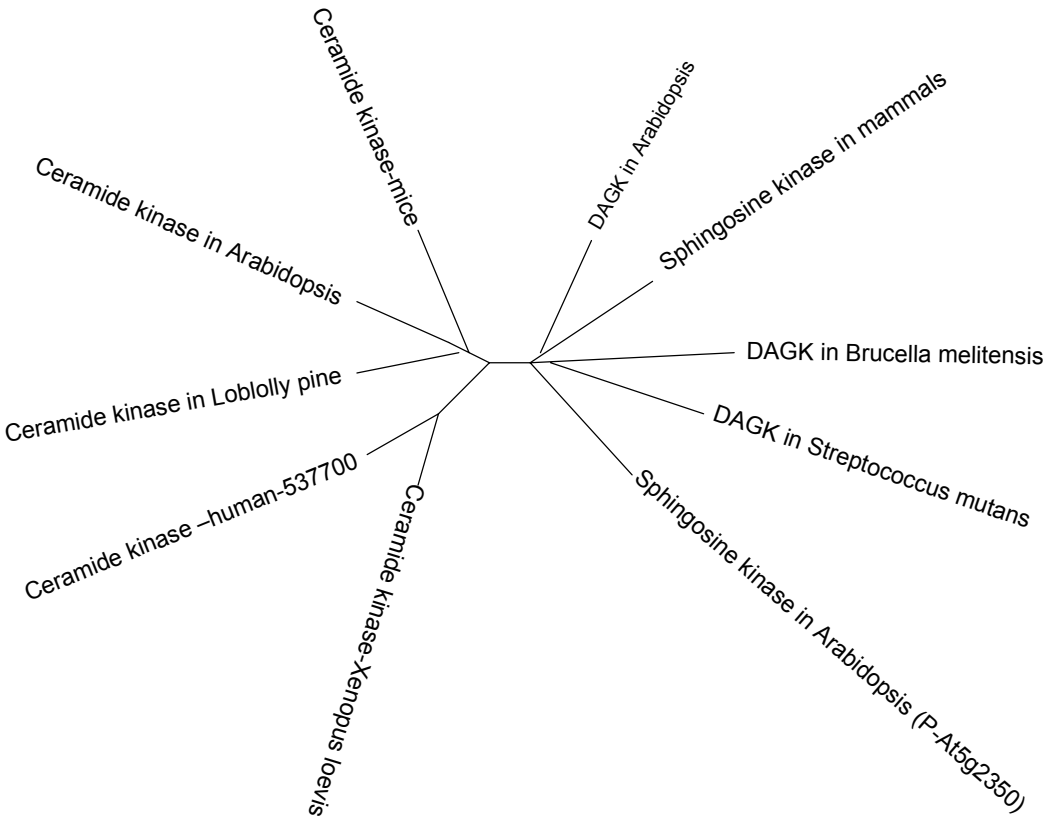


Figure 5.9. Phylogenetic analysis of ceramide kinases, diacyglycerol kinases and sphingosine kinases in various organisms
Various organisms: ceramide kinase in Xenopus laevis(NP-001086227), ceramide kinase in Arabidopsis (AAQ62904), ceramide kinase in Rice (BAD25337), ceramide kinase in LP(Loblolly pine), ceramide kinase in human (NP-073603), DAGK in Arabidopsis (NP-193885), DAGK in Brucella melitensis (NP-540891), DAGK in Streptacoccut mulans (NP-721953), Sphk in Arabidopsis (NP-568432, Sphk=Sphingosine kinase), Sphk2 in mammal (EAW52365).

Based on my results, I searched the NCBI database for alternative versions of CERK in different organisms. Table 5.1 also shows multiple versions of ceramide kinase exist in many organisms. The highly conserved motifs, which are closely related to the phosphorylation reaction, are also conserved in different organisms, although these have not been discussed in the literature.

5.4. Conclusions and significance

Ceramide kinase is actively present in embryos during embryo development of Loblolly pine. Both cloned versions, Pt-CERKL and Pt-CERKS, have catalytic function to convert ceramide to ceramide 1 phosphate. Pt-CERK enzymes might be less dependent on Ca^{2+} like their homology *h*CERK. Also, the microsomal membranes are not necessary for Pt-CERK enzyme phosphorylation reactions in *vitro*, this is consistent with previous descriptions of CERK as being "membrane associated' but cytoplasmic located, which suggested that the membrane system might only function to localize substrates or localize interacting enzymes to the same location for catalytic reaction in vivo. Three versions of Pt-CERKs are expressed in embryogenesis in different patterns and might respond to various environmental or hormones stimulus. Protein structure analysis of the three versions suggests that difference exists but all have the phosphorylation functions. The phosphorylation function was also be highly conserved through evolution; specifically the catalysis related domains were conserved from lower organism to human. Bioinformatics is thus a very strong and useful tool in molecular biology to illuminate potential function by comparisons with homologous genes.

Embryogenesis is bioactive process, which needs frequently to turn on and shut off certain signals to control gene expression and further mediate cell growth, proliferation, survival and death. Because ceramide-1-phosphate is interconvertible with the bioactive sphingolipid metabolites, ceramide, sphingosine, and sphingosine-1-phosphate, it has been known be involved in regulation of important physiological events by controlling cell survival and cell death (Merrill et al. 1997; Mitsutake & Igarashi 2005). So the conversion of ceramide to ceramide-1-phosphate may serve as a mechanism to mediate

cell apoptotic and cell survival process based on specific pathologic situation and various cell growth requirements during embryogenesis process in Loblolly pine.

Chapter 6: Overall conclusions and recommendations

6.1. Overall conclusions

BLAST searching against the pine cDNA library for the homologs of the sphingolipid metabolism enzymes in Arabidopsis reveals that many enzymes in the sphingolipid metabolism pathway have homologues in Pine with significant E-value. Results suggested that sphingolipids might be active players in embryogenesis of Loblolly pine. Further study of two key sphingolipid metabolism genes: serine palmitoyltransferase and ceramide kinase in embryos of Loblolly pine suggested that may sphingolipids play vital roles in embryo development in a highly conserved way like sphingolipids and their metabolism enzymes in other organisms.

Two SPT1 and SPT2 like cDNA sequences were cloned from embryos of Loblolly pine. Analysis in the nucleotides sequences and their coding protein sequences demonstrated that these two cDNA may code for the two subunits of serine palmitoyltransferase I and serine palmitoyltransferase Ⅱ in Loblolly pine. The catalytic domains in both subunits were highly conserved compared to its homologs in yeast, Arabidopsis, and human with significant E value. The motif 'GTFTKSFG', which is the pyridoxal phosphate-binding region in SPT2 of Arabidopsis, yeast, human and even drosophylia exist in Loblolly pine in exact the same sequence. So this motif in Loblolly pine most likely has the same function as in Arabidopsis. Multiple transcripts of SPT1 exist in Loblolly pine, one encode a 484 a. a protein with the catalytic domain; the others encode non-catalytic proteins. The differences among those transcripts are just variable in nucleotide length loss, so it was suggested that the multiple transcripts probably are alternative splicing results. If translated, these SPT1 proteins may be dominant negative

isoforms and associaties with other proteins to form non-functional complexies. Alternatively, SPT1 forms may stimulate additional pathways. The multiple SPT1 transcripts could play different roles in development.

Three different versions (Pt-CERKS, Pt-CERKM and Pt-CERKL) of ceramide kinases were found in embryo of Loblolly pine. Domain analysis by bioinformatics found the short version Pt-CERKS lost a calcium/calmodulin domain and a ZnF-C4 motif compared to the long version, Pt-CERKL While the middle version Pt-CERKM lost the PH binding domain compared to the long version, Pt-CERKL and short version Pt-CERKs. Like other DAGK family, CERK possesses a DAGK like catalytic domain located in residue 195 to 472 with high e value, $6.6e^{-15}$. A highly conserved ATP binding region in DAGK superfamily also presents in residue 260-271 of Pt-CERKl and Pt-CERKs in the same pattern as GXGXXXG. Three versions showed distanct expression pattern during embryogenesis process.

The short version Pt-CERKs is expressed in early stages, and some times in late stages. While the potential Pt-CERKM does not show up in 2005 embryos and only presents in 2004 embryos. Long version, Pt-CERKL demonstrates a constant expression in zygotic embryogenesis and exhibits a gradually increased pattern in somatic embryogenesis, but short and middle versions do not show in somatic embryogenesis. So expression pattern suggests that short and middle version Pt-CERK proteins might be finely regulated by embryo development mechanism together with environmental stimulis.

Enzyme assay for Pt-CERKL and Pt-CERKS and a truncated short peptide, which contains only the catalytic domain, indicated that ceramide kinases in Loblolly pine may

have the catalytitc function same as their homologs in Arabidopsis and human to phospharylate ceramide to form ceramide 1 phosphate. The catalytic function of the truncated short peptide further proves that the catalytic domain is the DAGK domain. Furthermore, as in ceramide kinase in mammals, ceramide kinase in Loblolly pine most likely less dependent on Ca^{2+} ion for their catalytic reaction. But different to the ceramide kinase in Arabidopsis, which is Ca^{2+} dependent. The vital microsomal functions were not necessary for the ceramide kinase catalytic reaction *in vitro* for Pt-CERK enzymes in Loblolly pine.

With those new tools to study CERK, it will be possible to explore its possible role in cell protection, dead cell clearing, and cell survival. So our new findings, the three Pt-CERK proteins might be involved in the three type activities, membrane fusion, mitogenic effects and phogocytosis in concert.

6.2. Recommendations for future work

Although the significant effects of sphingolipids in plant cell growth, cell apoptosis, and plant phagocytosis have been investigated, general outlines about sphingolipids in plants have been achieved. However, the functions of all metabolic enzymes and their products are still under development.

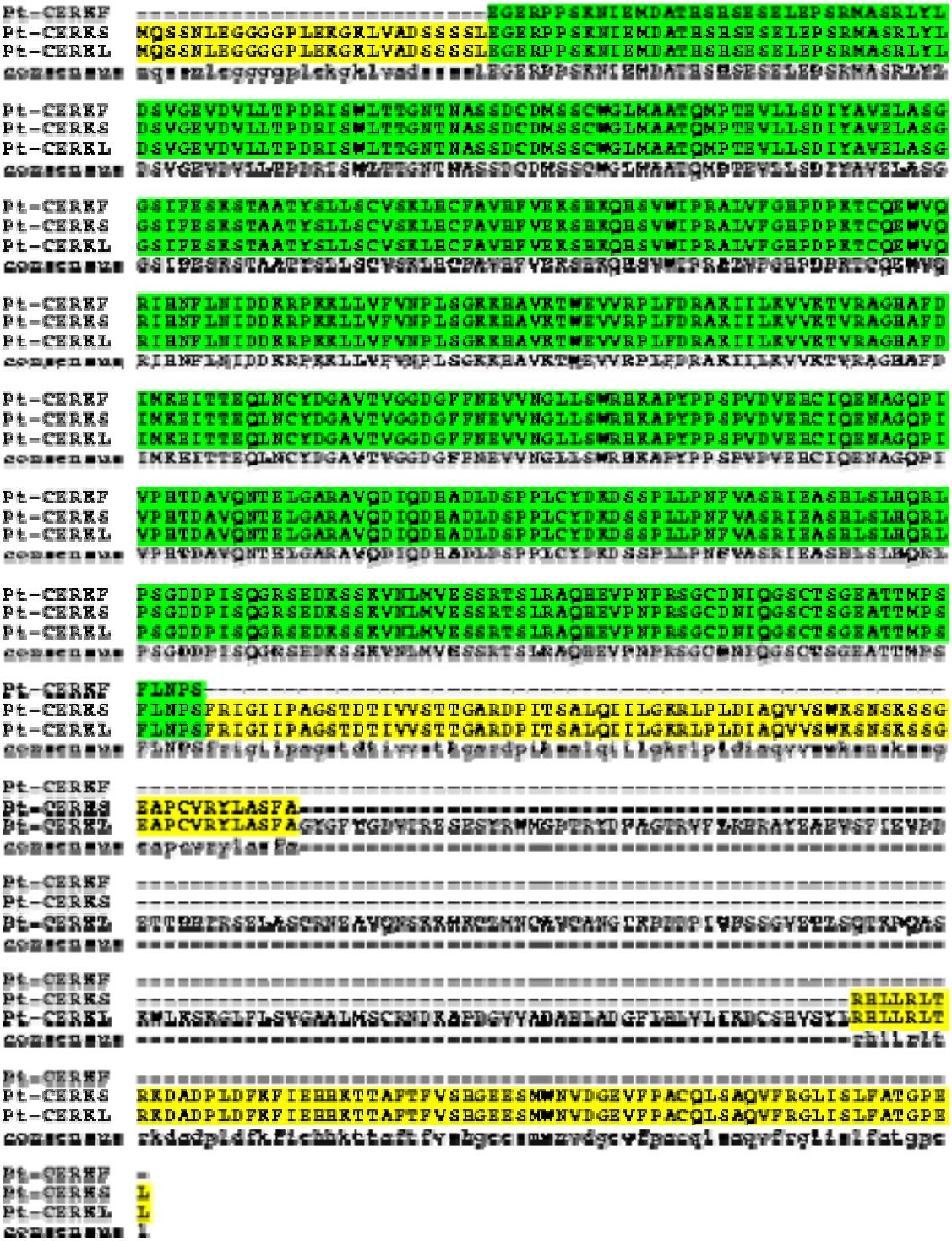
This study was the first investigation of sphingolipid metabolic enzymes in a gymnosperm. The encouraging results suggested that sphingolipids may be active players in gymnosperm and might be functioning in a highly conserved way like their homologs in other organisms. However, the specific metabolism of all the metabolic enzymes and the small active sphingolipids are still unclear and need further investigations.

Refer to serine palmitoyltransferase and ceramide kinase in Loblolly pine, the following aspects need more research:

1. Future research will focus on Pt-SPT function analysis to prove those two putative mRNAs coding two subunits of SPT in Loblolly pine and those two subunits work as a heterodimers.
2. Investigate the functions of severals cloned transcripts of Pt-SPT1 and their cellular locations.
3. Do genomic analysis to study if the above multiple transcripts arise from alternative splicing results and further to check the promoter regions for two full lengths Pt-SPT1 and Pt-SPT2 and their transcription control mechanisms.
4. Conduct specific research about the cloned small transcript, which code a protein with only 90 a. a. and its sub-cellular location.
5. Continue to clone the full length of the middle version Pt-CERKm.
6. A biochemical investigation for the PH, Calcium/calmodulin, ZnF-C4 motif and the residue Glycine in Pt-CERK enzymes to illuminate the function similarity and difference among the three versions of Pt-CERK enzymes.
7. Do genomic walking to check if the three versions are alternative splicing results and examine the promoter region of Pt-CERK and clarify their transcription modification.
8. Use cultured cell line to evaluate the plant hormone control to sphingolipids level during embryogenesis of Loblolly pine.
9. Display the activity difference of serine palmitoyltransferase and ceramide kinase in somatic and zygotic embryogenesis.

10. Based on a complete investigation in serine palmitoyltransferase and ceramide kinase in zygotic embryogenesis of Loblolly pine to establish a serine palmitoyltransferase active model then contrast to the serine palmitoyltransferase and ceramide kinase activities in somatic embryogenesis to figure out abnormal track of these two genes expression in somatic embryogenesis.

APPENDIX 1: Alignment of overexpressed Pt-CERK proteins



APPENDIX 2: Materials and methods

A.1. Embryos

Fresh Loblolly pine embryos in 2004 and 2005 were obtained from the cones provided by Weyerhaeuser Company (for zygotic embryos) and the lab cultured cells (for somatic cells).

A.1.1. Zygotic embryo tissue collection

Zygotic embryos tissues were collected from mother tree 7-56 and 500 and 512 from June 20th to September 15th in 2004 and 2005. Loblolly pine cones were collected, packaged with ice and shipped to the lab by Weyerhaeser Company. Seed were dissected and examined under a microscope for development stages, flash frozen in liquid N_2, and stored at -70 ^{o}C prior to using.

A.1.2. Somatic embryo tissue collection

Somatic embryo cultures were conducted in the IPST Forestry Biology Group. First the intact ovule containing the dominant immature zygotic embryo (~ stage 2) and subordinate embryos were collected from Loblolly pine cones. The collected intact ovule were put in the medium # 1959 (Table A.1) for initiation. In the initiation medium, each intact ovule will extruded many subordinate zygotic embryos.

Table A.1. Loblolly pine embryos maintenance medium # 1959

INSTITUTE OF PAPER SCIENCE AND TECHNOLOGY					
Cell and Tissue Culture Laboratory		Log#			
		Label as		1959	
		Volume		1L	
		Carbohydrate		sucrose	
Purpose:		Made by:		Date:	
Reference R.P.#:		Gelling Agent %:			
ADD COMPONENTS IN ORDER LISTED					
COMPONENT	STOCK	A/FS	AMOUNT	ADDED/L	STOCK AMOUNT
1/2 P6 MACRO (-)	50X	A	20	ml	
NH_4NO_3	0.1g/ml	A	2	ml	
1/2P6MICRO	100x	A	10	ml	
$CuSO_4.H_2O$	100x	A	3.8	ml	
$ZnSO_4.7H_2O$	10mg/ml	A	28.8	ml	
FeEDTA	100X	A	5	ml	
$AgNO_3$	1mg/ml	A	3.4	ml	
WPM VITAMINS	1000x	A	1,000	ml	
GLYCINE	1.0mg/ml	A	2	ml	
NAA	1.0mg/ml	A	2	ml	
BAP	1mg/ml	A	630	ul	
KINETIN	1mg/ml	A	610	ul	
Folic Acid	1.0mg/ml	A	0.5	ml	
Biotin	0.1mg/ml	A	0.5	ml	
MES	0.1g/ml	A	2.5	ml	
INOSITOL	SOLID	A	20	g	
Maitose	SOLID	A	15	g	
CASAMINO Acid	SOLID	A	0.5	g	
Activated Charcoal	SOLID	A	0.05	g	
Gelrite	SOLID	A	2.0	g/500ml	
Following components to added after autoclaved					
GLUTAMINE	50mg/ml	FS	9	ml	
Br	1mg/ml	FS	48.08	ul	
Alpha-Ketoglutarate	20mg/ml	FS	5.0	ml	
Vitamin B12	0.1mg/ml	FS	1.0	ml	
Vitamin E	5mg/ml	FS	20.0	ul	
					pH5.7

Note: WPM =Woody plant medium, BAP=benzylaminopurine, NAA= a-naphthalene acetic acid,

Table A.2. Loblolly pine embryos maintenance medium # 1250

INSTITUTE OF PAPER SCIENCE AND TECHNOLOGY					
Cell and Tissue Culture Laboratory		Log#			
		Label as		1250	
		Volume		1L	
		Carbohydrate		sucrose	
Purpose:	Initiation	Made by:		Date:	
Reference R.P.#:		Gelling Agent %:			

ADD COMPONENTS IN ORDER LISTED					
COMPONENT	STOCK	A/FS	AMOUNT	ADDED/L	STOCK AMOUNT
1/2 P6 MACRO (-)	50X	A	20	ml	
$NH4NO_3$	0.1g/ml	A	6.038	ml	
1/2P6MICRO	100x	A	10	ml	
FeEDTA	100x	A	2.5	ml	
WPM VITAMINS	1000x	A	1	ml	
GLYCINE	1.0mg/ml	A	2	ml	
2,4-D	10mg/ml	A	0.11	ml	
BAP	1mg/ml	A	0.45	ml	
KINETIN	1mg/ml	A	0.43	ml	
Folic Acid	1.0mg/ml	A	0.5	ml	
Biotin	0.1mg/ml	A	0.5	ml	
MES	0.1g/ml	A	2.5	ml	
INOSITOL	SOLID	A	1	g	
SUCROSE	SOLID	A	30	g	
CASAMINO Acids	SOLID	A	0.5	g	
	1	bottles			
separate into	0.5	L each			
eGelrit	solid	A	2.5	g	
GLUTAMINE	50mg/ml	FS	9	ml	
ABA	10mg/l	FS	0.133	ml	
					pH5.7

Note: WPM=Woody plant medium, 2, 4 D=dichlorophenoxyacetic acid, BAP=benzylaminopurine, ABA=abscisic acid, A=autoclaving, FS=filter-sterilized. ABA and Glutamine should be added after autoclaving.

Then the extrudes subordinate embryos were maintained in the maintenance medium # 1250 (Table A.2), in which the embryos can be multiplied to generate thousands of embryos in same developmental stages; or the embryos can also be maintained in a 45 ml

volume liquid medium # 1133 (Table A.3). Every seven days, aliquots of liquid suspension cultures (LSCs) were sub-cultured in fresh medium #1133. The maintenanced early stage embryo s were plated in medium (#1562) (Table 4.4) for embryos development and maturation. The development process could last 4-5 weeks; different stage embryos were collected during this process.

Table A.3. Loblolly pine embryos maintenance medium # 1133

INSTITUTE OF PAPER SCIENCE AND TECHNOLOGY					
Cell and Tissue Culture Laboratory		Log#			
		Label as		1133	
		Volume		1L	
		Carbohydrate		sucrose	
Purpose:		Made by:		Date:	
Reference R.P.#:		Gelling Agent %:			
ADD COMPONENTS IN ORDER LISTED					
COMPONENT	STOCK	A/FS	AMOUNT	ADDED/L	STOCK AMOUNT
1/2 P6 MACRO (-)	50X	A	20	ml	
NH_4NO_3	0.1g/ml	A	6.038	ml	
1/2P6MICRO	100x	A	10	ml	
FeEDTA	100x	A	2.5	ml	
WPM VITAMINS	1000x	A	1	ml	
GLYCINE	1.0mg/ml	A	2	ml	
2,4-D	10mg/ml	A	0.11	ml	
BAP	1mg/ml	A	0.45	ml	
KINETIN	1mg/ml	A	0.43	ml	
Folic Acid	1.0mg/ml	A	0.5	ml	
Biotin	0.1mg/ml	A	0.5	ml	
MES	0.1g/ml	A	2.5	ml	
INOSITOL	SOLID	A	1	g	
SUCROSE	SOLID	A	30	g	
CASAMINO Acids	SOLID	A	0.5	g	
GLUTAMINE					
ABA	50mg/ml	FS	9.0	ml	
	10MG/L	FS	0.133	ml	pH5.7

Note: WPM=Woody plant medium, 2, 4 D=dichlorophenoxyacetic acid, BAP=benzylaminopurine, ABA=abscisic acid, A=autoclaving, FS=filter-sterilized. ABA and Glutamine should be added after autoclaving.

Table A.4. Loblolly pine embryo development and maturation medium #1562

INSTITUTE OF PAPER SCIENCE AND TECHNOLOGY					
Cell and Tissue Culture Laboratory		Log#			
		Label as		1562	
		Volume		1L	
		Carbohydrate		sucrose	
Purpose:		Made by:		Date:	
Reference R.P.#:		Gelling Agent %:			
ADD COMPONENTS IN ORDER LISTED					
COMPONENT	STOCK	A/FS	AMOUNT	ADDED/L	STOCK AMOUNT
1/2 P6 MACRO	50X	A	20	ml	
MAT	100x	A	10	ml	
1/2P6MICRO	100x	A	15	ml	
MAT	1000x	A	1	ml	
FeEDTA	1.0mg/ml	A	2	ml	
WPM VITAMINS	100X	A	10	ml	
GLYCINE	Solid	A	20	g	
INOSITOL	Solid	A	0.5	g	
MALTOSE	Solid	A	130	g	
CASAMINO ACIDS					
Acro PEG-8000	1	L			
PHYTAGEL	Solid	A	2.5		
ABA	10mg/ml	FS	520		
GLUTAMINE	50mg/ml	FS	9		
					pH5.7

Note: PEG-8000 = polyethylene glycol MW of 8000

A.2. Other materials

RNeasy Plant Mini Kit for RNA isolation was purchased from *QIAGEN*. DNA polymerase, JM 109 competent cells, PCR, pGEM-T Easy vectors, SMART™ RACE cDNA Amplification Kit and PCR and gel purification kits was obtained from *Clontech* and *Promega*. 4-20% polyacrylamide gels, nitrocellulose and other western blotting kits

were purchased from *Bio-Rad*. Restriction enzymes, NheI/XhoI and NdeI were obtained from *New England Nuclear.* EcoRI was obtained from *Promega*.

A.3.Methods

A.3.1 Clone full length Pt-SPTs and Pt-CERKs

RNA isolation

Total RNA was isolated from chosen stage 7 and stage 9.1 embryos (7-56) using plant material specific Kit from *QIAGEN* (Qiagen 2006).

To obtain optimal RNA yield and purity with RNeasy columns, it needs to begin with the correct amount of plant material. Generally, a maximum of 100 mg plant material or $1x10^7$ cells can be processed with Rneasy mini columns. For most plant material, the binding capacity of the column and the lysating capacity of Buffer RLT will not be exceeded by these amounts.

In this study, lysis buffer: RLT was used. β-Mercaptoethanol must be added to Buffer RLT. Because β-Mercaptoethanol was toxic, it was necessary to dispense in fume hood and wear appropriate protective clothing. 10ul β-Mercaptoethanol per 1ml Buffer RLT was added. Buffer RLT was stable for 1 month after addition of β-Mercaptoethanol.

Note:

a. Generally, Dnase digestion is not required since the Rneasy silica-membrane technology efficiently removes most of the DNA without Dnase treatment. Buffer RLT may form a precipitate upon storage. If necessary, re-dissolve by warning, and then place at room temperature.

b. Buffer RLT, Buffer RLC, and Buffer RW1 contain a guanidine salt and are therefore not compatible with disinfecting reagents. Guanidine is an irritant. Take appropriate safety measures and wear gloves when handling.

c. All steps of the Rneasy protocol are performed at room temperature. During the procedure, worked quickly.

d. All centrifugation steps are performed at 20-25 ^{o}C in a standard microcentrifuge. Ensure that the centrifuge does not cool below 20 ^{o}C.

Procedures:

1. Determined the amount of plant material by weighing tissue. Do not use more than 100 mg. 100 mg was used in this study.
2. Immediately placed the weighted sample in liquid nitrogen, and grinded thoroughly with a mortar and pestle. Decanted powder and liquid nitrogen into an Rnase-free, liquid-nitrogen-cooled, 2 ml microcentrifuge tube. Allowed the liquid nitrogen to evaporate, but do not allow the tissue to thaw.

   Note: RNA in plant material is not protected after harvesting until the sample is flash frozen in liquid nitrogen. Frozen tissue should not be allowed to thaw during handling. The relevant procedures should be carried out as quickly as possible.
3. Added 450 µl Buffer RLT to a maximum of 100 mg tissue powder. Vortexed vigorously. Short (1-3 min) incubation at 56 ^{o}C may help to disrupt the tissue. However, for samples with high starch content, incubation at elevated temperatures should be omitted in order to prevent swelling of the starting material.

4. Pipeted the lysate directly onto a QIAshredder spin column (lilac) placed in 2 ml collection tube, and centrifuged for 2 min at maximum speed. Carefully transferred the supernatant of the flow-through fraction to a new microcentrifuge tube without disturbing the cell-debris pellet in the collection tube. Used only this supernatant in subsequent steps.
5. Added 0.5 volume ethanol (96-100%) to the cleared lysate, and mixed immediately by pipetting (Do not centrifuge). Continued without delay with next step.
6. Applied sample, including any precipitate that may have formed, to an RNeasy mini column placed in a 2 ml collection tube. Closed the tube gently, and centrifuged for 15 seconds at greater than 8000 g. Discarded the flow-through.
7. Added 700 µl Buffer RW1 to the RNeasy column. Closed the tube gently, and centrifuged for 15 seconds at greater than 8000 g to wash the column. Discarded the flow-through and collection tube.
8. Transferred the RNeasy column into a new 2 ml collection tube. Pipeted 500 µl Buffer RPE onto the RNeasy column. Closed the tube gently, and centrifuged for 15 seconds at greater than 8000 g to wash the column. Discarded the flow-through.
9. Added another 500 µl Buffer RPE to the RNeasy column. Closed the tube gently, and centrifuged for 2 min at greater than 8000 g to dry the RNeasy silica-gel membrane. Continued directly with next step.

10. To elute, transferred the RNeasy column to a new 1.5 ml collection tube. Pipeted 30-50 μl RNase-free water directly onto the RNeasy silica-gel membrane. Closed the tube gently, and centrifuged for 1 min at 8000 g to elute.
11. If the expected RNA yield was greater than 20 g, repeated the elution step as described with a second volume of RNase-free water. Eluted into the same collection tube.

Specific gene cloning

The homologs (EST in pine cDNA library) obtained from the preliminary analysis were used to design the gene specific primers for the 5'-RACE-Ready cDNAs and 3'-RACE-Ready cDNAs with program –*Primer 3 INPUT as following:* The 5' RACE Gene specific primer for SPT1 (5'-TGGGTCCCTGAGCCACTACATCCA-3'), 3' RACE Gene specific primer for SPT1 (5'-TGCTGGTATGGGGTATGCTTTGGCA-3'); The 5'RACE Gene specific primer for SPT2 (5'-TTGCCGTTTTTCAGCACAACACACC-3'), the 3'RACE Gene specific primer for SPT2 (5'-TGCAGGAAAGGCAACTGTCACCACTGC-3'). The 3'RACE Gene specific primer for ceramide kinase in Loblolly pine (5'-CCAATCCGAGATCGGGATGTGAT-3'). The 5'RACE Gene specific primer for ceramide kinase in Loblolly pine (5'-GAACCCCCACTAGCAAGCTCCACAGCA-3')

BD SMART RACE Kit included a protocol for the synthesis of two separate cDNA populations: 5'-RACE-Ready cDNA and 3'-RACE-Ready cDNA. The cDNA for 5'-RACE was synthesized using a modified lock-docking oligo (dT) primer and the BD SMART Π An oligo. The modified oligo (dT) primer, termed the 5'RACE CDS Primer

(5'-CDS), had two degenerate nucleotide positions at the 3'end (Barnes 1994). Followed the kit manual for detail procedures (Qiagen 2006).

The forward and reverse primers were designed based on the 5' cDNA sequence and 3'cDNA sequence of SPT in Loblolly pine as following: Pt-SPT1F (5'-TCCCGCAGCACACCCTTGACCA-3'); Pt-SPT1R (5'-TTGCCTGACCTAGCTGAACATGAATGA-3'), Pt-SPT2F (5'-CGAATGCGTGCAAGCTTTTGAGCTCCT-3') and Pt-SPT2R (5'-CGCCACATCAAAATGTGGTGCCATTC-3'). The above primers were used to run PCR to clone full length Pt-SPT1 and Pt-SPT2. The forward and reverse primers were designed based on the 5' cDNA sequence and 3'cDNA sequence of CERKs in Loblolly pine as following: Pt-CERKF (5'-AGAGGGCGGGGACGTCCATCCA-3'); Pt-CERKR (5'-TGTGCGCTTCCCCTCCCAATA-3'). This pair of primers was used to run PCR to clone full length Pt-CERKs.

The ligation used pGEM-T Easy Vector Systems. The pGEM-T Easy Vector Ligation Reaction was used to ligate the target cDNA sequences to pGEM-T Easy vector then be transformed to *JM 109* competent *E.coli* cells. PCR was used to amplify Pt-SPTs and Pt-CERKs fragments. Then the plasmid with Pt-SPTs and Pt-CERKs insertion were sent for sequencing.

A.3.2. Protein overexpression

Construction of expression vector

Gel electrophoresis, restriction enzyme digestion, ligation of DNA and transformation of *E.coli* were performed according to the protocols from Clontech Laboratories, Inc. and Novagen kit manuals in the following pET vector express system.

The CERK long version (Pt-CERKL) and CERK short version (Pt-CERKS) full-length cDNAs were obtained by PCR amplification on the plasmid pGEM-T Easy vector with primers Pl-F-CERK(5'-CTAGCTAGCTTCGAAGGTAACGTTCTGAGCAACAA3'); Pl-R-CERK (5'-CCGGAATTCTTGGAGGTGACCCCGGATTTAAACA-3'); A short fragment of Pt-CERKS (Pt-CERKF) was obtained by PCR amplification on the stored plasmid pGME T Easy vector with primers Ps-F-CERK(5'-CTAGCTAGCGAGGGGGAAAGACCCCCGTCTAA-3') and Ps-R-CERK(5'-CCGGAATTCTGATGGATTCAGGAAAGGAGGGCATAG-3'). Using above primers, a NheI restriction site was introduced to the 5'-end of Pt-CERK cDNA sequences and an EcoRI restriction site was introduced to the 3'-end of Pt-CERK cDNA sequences. A 1798bps DNA (Pt-CERKL) fragment; a 1385 bps DNA (Pt-CERKS) fragment and a 1218bps fragment of Pt-CERKS (Pt-CERKF) were produced with above primers. PCR fragment Pt-CERKs were purified by agarose gel electrophoresis, digested with NheI/EcoRI, and ligated into the NheI/EcoRI sites of pET28a+ to produce plasmid pET28-Pt-CERK-L, pET28-Pt-CERK-S and pET28-Pt-CERK-F. The final recombinant plasmids were characterized by restriction enzyme digestion and regions around the cloning sites was re-sequenced to ensure that the desired constructions were prepared correctly.

The pET Expression System

The pET System (Figure A.1) was the most powerful system developed for the cloning and expression of recombinant proteins in *E. coli*. This expression system was developed in 1986 by Studier and Moffatt (Moffatt & Studier 1986). In this system, Moffatt created an RNA polymerase expression system that was selective for

bacteriophage T7 RNA polymerase. The expression system had two different methods in joining T7 RNA polymerase into the cell. First a lambda bacteriophage was used to insert the gene which codes for T7 RNA polymerase, and then the gene for T7 RNA polymerase was transferred into the host chromosome (Moffatt & Studier 1986).

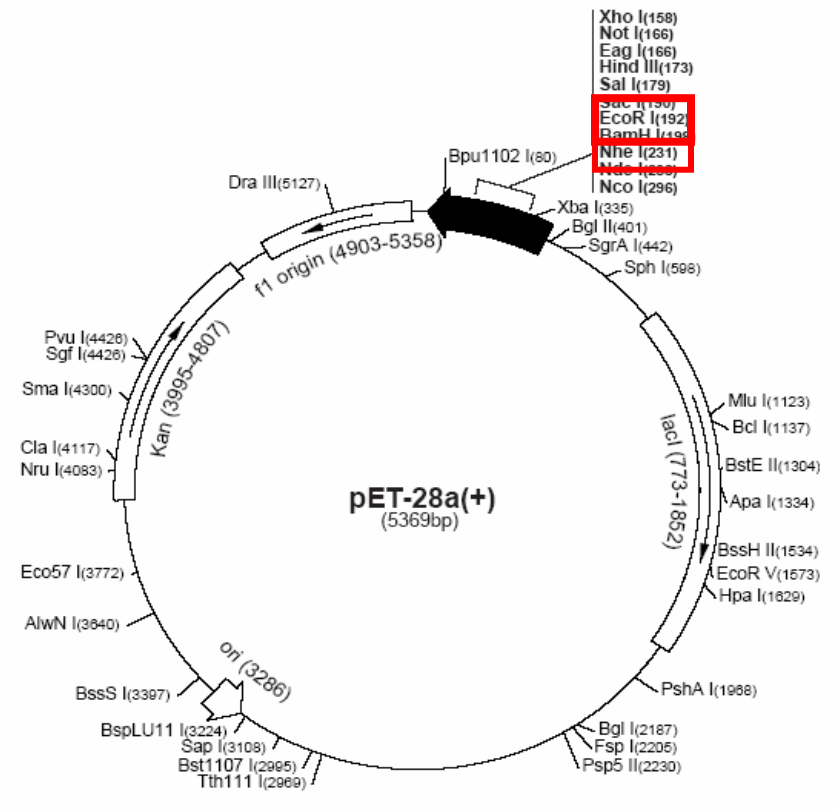


Figure A.1. The pET vector

The pET-28a-c (+) vectors carry an N-terminal His•Tag®/thrombin/T7•Tag® configuration plus an optional C-terminal His•Tag sequence. Unique sites are shown on the circle map. Note that the sequence is numbered by the pBR322 convention, so the T7 expression region is reversed on the circular map. The cloning/expression region of the coding strand transcribed by T7 RNA polymerase is shown below. The f1 origin is oriented so that infection with helper phage will produce virions containing single-stranded DNA

that corresponds to the coding strand. Therefore, single stranded sequencing should be performed using the T7 terminator primer (Cat. No. 69337-3) from *Novagen.*

With control of bacteriophage T7 transcription and translation signals, target genes were cloned in pET plasmids. Expression was induced by providing T7 RNA polymerase in the host cell. T7 RNA polymerase was well known for it's highly selective and activity. Almost all of the cell's resources could be converted to target gene expression in this pET system; the target proteins could reach more than 50% of the total cell protein just a few hours after induction. Furthermore, this system could maintain target genes transcriptionally silent under the uninduced situation. Target genes were initially cloned using hosts (JM 109 *E.coli*) that do not contain the T7 RNA polymerase gene.

It is known that production of recombinant proteins have the potential harm to the host cell**,** which would eliminate plasmid instability. When the expression system was established in a nonexpression host, plasmids were then transferred into expression hosts (DE3) cont, which contained a chromosomal copy of the T7 RNA polymerase gene under the control of *lacUV5*, and expression would be induced by the addition of IPTG. The map of pET28a+ vector was shown in Figure A.1.

Selecting host strains

The *E.coli* (A λDE3 lysogen) B strain BL21 is the most widely used host for target gene expression. Strain BL21 lacks the *ompT* outer membrane protease that can degrade proteins during purification (Grodberg & Dunn). Thus, some target proteins should be more stable in BL21 than that in those host strains, which contain these proteases. However, there is a possibility to inhibit transcription by the host RNA polymerase,

because BL21 is sensitive to rifampicin. BL21 expression strains are supplied with the pET Systems commercially.

Several DE3 strains with various advantages were available commercially. Expression strain chosen would be based on each specific research purposes. For example, the thioredoxin reductase-deficient strains AD494 (DE3) and BL21*trxB* (DE3) were used to maximize soluble protein expression with the pET vectors. The *trxB*– cells had been shown to permit disulfide bond formation in the cytoplasm of *E. coli* (Derman et al. 1993), which also appeared to depend on the presence of an oxidized form of thioredoxin (Stewart et al. 1998).

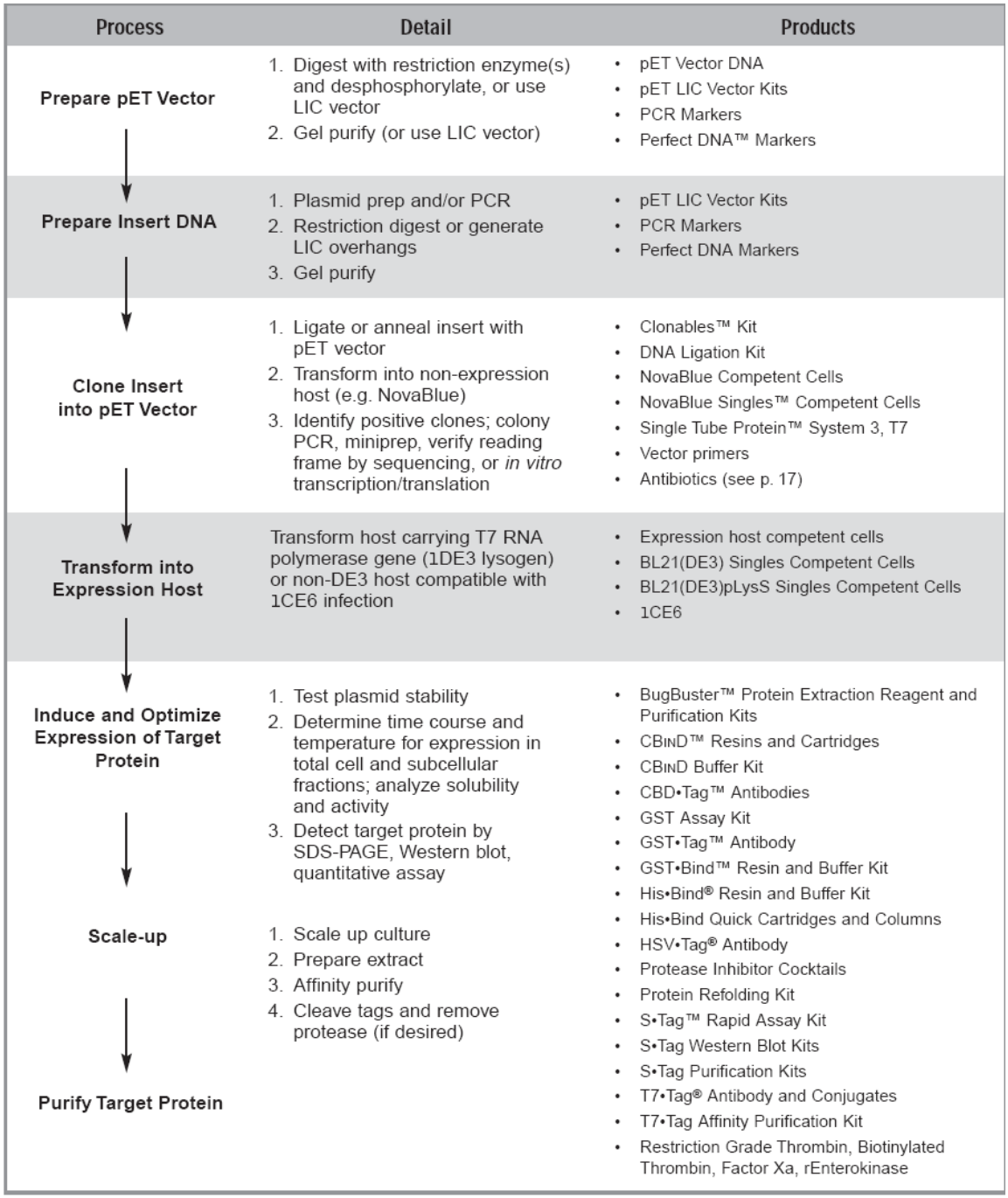


Figure A.2. pET system work process (*from Clontech Laboratories, Inc.)*

Since the *trxB* mutation in AD494 (DE3) and BL21*trxB* (DE3) is maintained by kanamycin selection, these strains are efficient for expression of target genes cloned in kanamycin resistant pET plasmids. Another DE3 lysogen that is used with the

NovaTope® System and which may provide additional advantages for other applications is NovaBlue (DE3). This NovaBlue strain has the *lacIq* repressor, which provides control over basal expression than the wild type repressor in the other strains. In this study, NovaBlue (DE3) was used as the expression strain.

The pET system process

The pET system process was described in Figure A.2.

Vector preparation

For vector preparation, used the restriction enzyme manufacturer's recommended buffer and incubation conditions for the enzymes.

To digest and gel-purify the vector

1. Assembled the following components in a microcentrifuge tube:

   3 μg pET vector

   3 μl 10X restriction enzyme buffer

   1 μl restriction enzyme EcoRI (12,000 μ/ml)

   1 μl restriction enzyme NheI (10,000 μ/ml)

   3 μl 1 mg/ml acetylated BSA (optional)

   x μl Nuclease-free water brought to volume

   30 μl Total volume

2. Incubated at 37°C temperature for 12 hrs.
3. Ran a 3 μl sample together with Perfect DNA Markers on an agarose gel to check the extent of digestion.

Three μg of a typical pET vector (5 kbp) corresponds to about 2 pmol DNA ends when linearized, or about 4 pmol ends if two enzymes were used for digestion.

Added gel sample buffer to the tral and load the entire sample into a large well for 45 μl on a 1% agarose gel containing 0.5 μg/ml ethidium bromides. Ran the gel far enough to separate the linear plasmid from nicked and supercoiled species. Uncut vector DNA was loaded in an adjacent lane to help distinguish undigested from linearized plasmid DNA.

4. Visualized the DNA band with a long wave UV light source and cut the band from the gel using a clean razor blade.
5. Cut the linearized plasmid DNA and purified with the QIAquik Gel Extraction Kit Protocol.
6. Stored the purified vector at –20°C till use.

Insert Preparation

Preparing inserts by restriction digestion followed by gel purification is simplification. PCR is usually used to isolate and/or amplify target genes for expression in pET plasmids. For this purpose, specific primers, which have restriction sites need be induced to this particular gene. Primers designing followed by four steps (1) isolate the translated portion of a cDNA sequence, (2) add convenient restriction enzyme sites, (3) add LIC overhangs, and (4) place the coding region in the proper reading frame.

When subcloned into the pET vectors with the PCR amplified cDNA fragments, it is necessary to gel purify the fragment of interest to remove the original plasmid, which will transform very efficiently. As little as 10 pg of contaminating supercoiled plasmid (*i.e.*,

less DNA than can be visualized on an agarose gel) could result in many more colonies containing the original plasmid than the desired pET subclone.

However, there is a potential risk in using PCR for insert preparation to introduce mutations. The error rate of the PCR reaction can be minimized in several ways:

- Use an enzyme with proofreading activity.
- Limit the number of PCR cycles.
- Increase the concentration of target DNA
- Increase the primer concentration

Cloning Inserts in pET Vectors

This process included ligation and transformation into a non-expression host, and analyzing results.

A. Ligation

Sample ligation protocol

1. Used DNA fragments with 2–4 base sticky ends, used 50-100 ng (0.015–0.03 pmol) of pET vector with 0.2 pmol insert (200 ng of a 2kb fragment) in a volume of 20 µl. Assembled the following components in a 1.5 ml tube (these components are available separately in the DNA Ligation Kit, from *Promega*). Added the ligase last.

   5 µl 2X Ligase Buffer (60mM TrisiHCL (pH 7.8), 20 mM $MgCl_2$, 20 mM DTT, 2 mM ATP, 10% PEG.)

   2 µl (50 ng/µl) prepared pET vector

   2 µl Prepared target gene insert (0.2 pmol)

   1 µl T4 DNA ligase, in: 10 mM Tris-HCl (pH 7.4), 50 mM KCl, 1 mM DTT.

0.1mM EDTA, 50% glycerol

Total volume: 10 µl

2. Added the ligase last, and gently mixed by stirring with a pipet tip. Incubate at 4°C overnight.

B. Transformation

Initial cloning was done in a *recA*– cloning strain, *PJM109,* to enable high percentage monomer plasmid yields for examination of the construct sequence, as well as separation of cloning from expression.

The strains described above for cloning and expression with pET vectors were prepared for transformation by standard procedures. Removed certain number of competent cell tubes from the freezer. Immediately placed the tubes into ice to make sure all but the cap was surrounded by ice. Also, placed the required number of empty 1.5 ml polypropylene microcentrifuge tubes on ice to pre-chill. Allow the cells to thaw on ice for ~5 min.

1. Visually checked the cells to see that they have thawed and gently flicked the cells 1–2 times to evenly resuspend the cells. Pipeted 50 µl aliquots of cells into each of the pre-chilled tubes.
2. Added 5 µl of a ligation reaction directly to the cells. Stirred gently to mix and returned the tube to the ice, making sure that the tube was surrounded by ice except for the cap. Repeated for additional samples.
3. Placed the tubes on ice for 20 min.
4. Heated the tubes for exactly 45 sec in a 42°C water bath; did not shake.

5. Placed the tubes on ice for 2 min.
6. Added 950 µl of room temperature SOC medium to each tube.

The outgrowth incubation was performed in a shaking incubator using a test tube rack anchored to the shaking platform. Placed each transformation tube in each room in the rack. During the outgrowth, placed the tubes at 37°C.

7. Spread 90 µl of each transformation on LB agar plates containing the kanamycin antibiotic. Used 30µg/ml kanamycin for vectors having the kanR gene.
8. Let the plates sit on the bench for several min to allow excess liquid to be absorbed, and then inverted and incubated overnight at 37°C.

A.3.3. Expression and purification of Pt-CERK proteins

The recombinant plasmids were transformed into *E.coli* strain BL21 for large-scale expression. Cells were grown at 37 ^{o}C with shaking in 2% LB broth (pH 7.4). When the turbidity (A_{600}) reached 1.0, IPTG was added to 0.4 mM and 0.8 mM, growth continued at 18 ^{o}C overnight and then the cells were harvested.

Use 5ml TALON® Single Step Column Purification. Follow the detail procedures described in the TOLON Mental Affinity Resins User Manual.

Procedure:

Expression Host Transformation

For transformation into an expression host (*i.e.,* a λDE3 lysogen), prepared appropriate competent λDE3 lysogen cells and used the same standard procedures described above. Streaked transformants for single colonies and prepared glycerol stocks (150 µl glycerol plus 350 µl cell culture).

Induction of λDE3 Lysogens

After a target plasmid was established in a λDE3, expression of the target DNA was induced by the addition of IPTG to a growing culture (Figure A.3). For pET constructions carrying the "plain" T7 promoter and with T7lac promoter, a final concentration of 0.4 mM IPTG and 0.8 mM was used in this study. An example of an induction protocol was presented below.

Preparation for Induction

Picked a single colony from a freshly streaked plate and inoculated 50 ml LB containing the appropriate antibiotic, kanamycin, in a 250 ml Erlenmeyer flask.

Alternatively, inoculated a single colony or a few microliters from a glycerol stock into 2 ml LB medium containing the appropriate antibiotic, kanamycin. Incubated with shaking at 37°C until the OD600 reaches 0.6–1.0. Stored the culture at 4°C overnight. Next morning, collected the cells by centrifugation (30 sec in a microcentrifuge). Re-suspended the cells in 2 ml fresh medium plus antibiotic, kanamycin and used this to inoculate 50 ml medium.

Sample Induction Protocol

1. Incubated with shaking at 37°C until OD600 reaches 0.4–1 (0.6 recommended; about 3 hrs).
2. Added IPTG from a 100 mM stock to a final concentration of 0.4 mM (T7 promoter) or 0.8 mM (T7*lac* promoter) and continued the incubation at 18◦C overnight).
3. Placed the flasks on ice for 5 min and then harvested the cells by centrifugation at 5000 × g for 5 min at 4°C.

Removed the supernatant and stored the cells as a frozen pellet at –70°C or continued with purification.

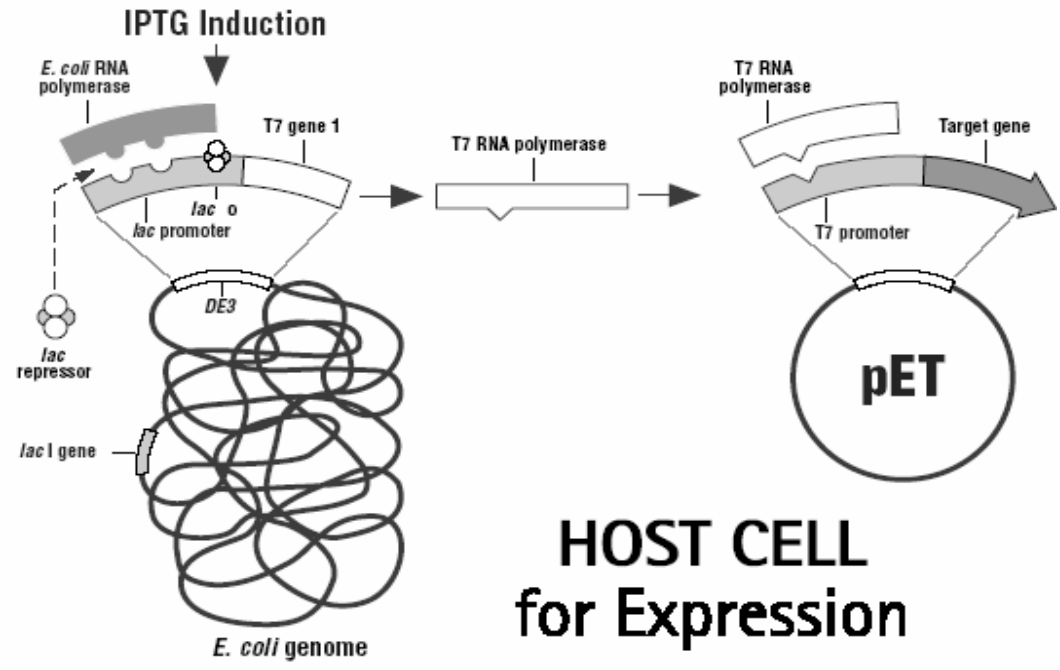


Figure A.3. Illustrattion of the host DE3 cell expression mechanism *(from Norgen)*

Protein purification using 5ml TALON Single Step Column Purification

The TALON® Single Step Columns were used with either gravity flow or centrifuge purification protocols.

Buffer Preparation and Additional Materials Required:

- 1X Equilibration/Wash buffer (50 mM Sodium Phosphate, 300 mM NaCl, pH 7.0)*

- Wash-2 Buffer (50 mM Sodium Phosphate, 300 mM NaCl, 7.5 mM Imidazole, pH 7.0)*
- 1X Elution buffer (50 mM Sodium Phosphate, 300 mM NaCl, 150 mM Imidazole, pH 7.0)*

* **Note:** These buffers were prepared by dilution of the buffers included in the TALON Buffer Kit (Cat. No. 635514).

Sample preparation and lysis:

Talon Single Step Columns were used for purification of any His-tagged protein from an *E. coli* culture (Figure A.4). For example, if screening transformants at an expression level picked a single colony from the plate and inoculated 4.7 ml of medium. Incubated the culture at 37°C until the OD_{600} reached ~0.6–0.8 AU. Then induced protein expression with the certain concentration of inducer agent which depended on specific expression strain and the expression plasmid being used. Continued to grow the culture with rigorous shaking at 37°C at 18◦C overnight [Optional: Remove 200 µl of the expression culture for SDS-PAGE analysis.]

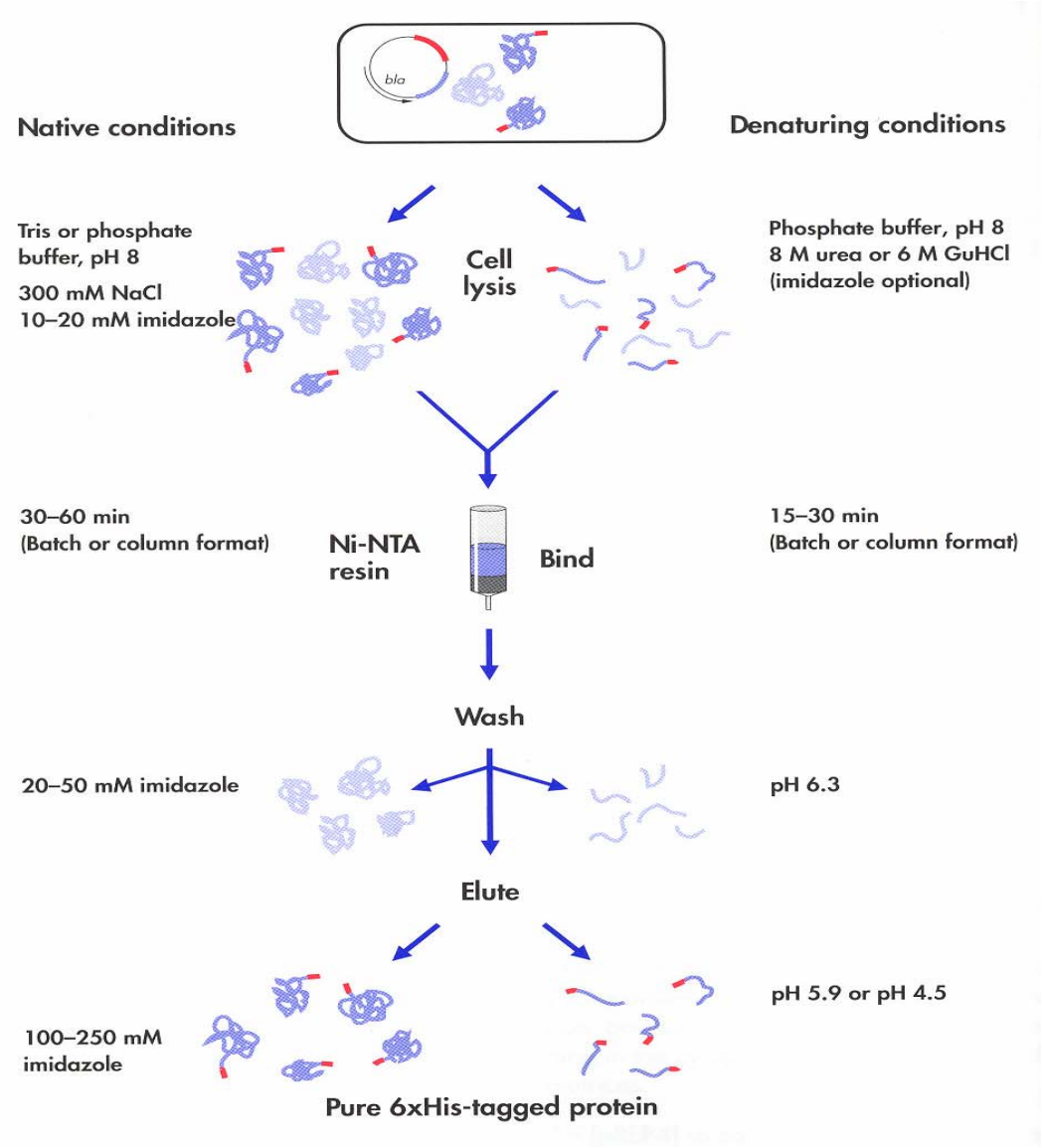


Figure A.4. Illustration of the 6XHis-tagged protein purification process *(From Clontech Laboratories, Inc)*

Procedure:

- Placed the bottom closure firmly on a Talon Single Step Column, removed the top cap, added 4.5 ml of culture and then replaced the top cap. Mixed the suspension

either on a shaker for 20–30 min at room temperature or by inverting the tube every 2 min for a total of 30 min.

- Removed the top cap and the bottom closure and placed the Talon Single Step Column into a Receiving Tube. Proceeded with either the gravity flow or the centrifuge procedure.

Centrifuge Procedure:

a. Centrifuged at 700 x g for 2 min. Took the column from the Receiving Tube and replaced the bottom closure [Optional: Removed 200 µl of non-adsorbed material collected in the Receiving Tube for SDS-PAGE and Protein Assay analysis].

b. Added 4.5 ml of 1X Equilibration/Wash Buffer, replaced the top cap and resuspend the resin by inverting the column. Removed the bottom closure and put the column into a Receiving Tube. Centrifuged at 700 x g for 2 min. Removed the column from the Receiving Tube and replaced the bottom closure [Optional: Remove 200 µl of Wash-1 for SDS-PAGE and Protein Assay analysis].

c. [Optional] For improved purity of target protein, repeat Step b twice.

d. Added 4.5 ml of Wash-2 Buffer, replaced the top cap and resuspended the resin by inverting the column. Removed the bottom closure and put the Talon Single Step Column into a Receiving Tube. Centrifuge at 700 x g for 2 min. Removed the column from the Receiving Tube and replace the bottom closure [Optional: Removed 200 µl of Wash-2 from the Receiving Tube for SDS-PAGE and Protein Assay analysis].

e. [Optional] For improved purity of the target protein, repeated the wash in Step d twice.

f. Added 1 ml of Elution Buffer and resuspended the resin by inverting the column for 2 min.

g. Removed the bottom closure and put the Talon Single Step Column into a Receiving Tube. Centrifuged the column in the tube at 700 x g for 2 min. Proceeded with Step 4. Protein Analysis.

Protein Analysis:

Determined the amount of protein in a 1:10 dilution of the non-adsorbed fractions and in the (undiluted) elution fraction by performing a *Bio-Rad Protein Assay.* Analyzed the samples by SDS-PAGE to determine the purity of the target protein (Elution fraction).

A.3.4. Pt-CERKs activity assay

Ceramide kinase activity was measured according to published protocols (Bajjalieh et al. 1999). This was performed in total volumes of 100 µl by mixing 20 µl substrate NBD-C6-Ceramide micelles with 20 µl 5X reaction buffer (75 mM MOPS, pH 7.2, 250 mM NaCl, 15 mM $CaCl_2$, 2.5 mM dithiothreitol (DTT), 5 mM EGTA, pH 7.5), 54.9 µl purified Pt-CERK proteins and certain amount non-pure water to 100 µl and 54.9 µl purified Pt-CERK proteins. The reaction was initialed with addition of 10 µl 5 mM magnesium, 5 mM ATP and was incubated at 37 ^{o}C for 25 minutes. Reactions were stopped by adding 1.2 ml chloroform/methanol 1:1 and phases were broken by adding 530 µl of 1M KCl, 20 mM Mops, pH 7.2, vortexed 30s, followed by 5 min

centrifugation, 200 µl of the organic phase was moved and then lipids were extracted and separated on Silica Gel 60 high performance TLC plates using chloroform/methanol/water (25:20:1.1) as the solvent system. Bands corresponding to C1P were quantified using an imaging analyzer BAS2500 (Fuji Film).

A few alternation reactions were carried out to test Ca^{2+} effects in different Pt-CERK enzymes by preparation of different $CaCl_2$ concentration: 0 mM, 1.875 mM and 15 mM, of the 5X reaction buffer.

APPENDIX 3: Fujifilm luminescent image analyzer LAS-1000 plus system

The FUJIFILM Luminescent Image Analyzer LAS-1000plus uses a 1,300,000-pixel colling CCD camera developed exclusively by Fuji Film and is a one-unit image analysis system (Figure A.5) that can be used for fluorescent and enzyme amplification fluorescent methods, and mainly for the chemical fluorecscence method.

Figure A.5. LAS-1000 plus system hardware configure

1. Operations of Fujifilm Luminescent Image Analyzer LAS-1000plus System

   Startup

   Turn ON the power supply

   On the computer, activate the image reading software, “Image Reader LAS-1000.

2. Exposing Chemiluminescence Samples

   Set the CCD temperature on the computer.

   Set Operation Mode to Focusing on the computer

   Select the tray rail in accordance with the size of the sample.

   Adjust the focus using printed matter or the like as a stand-in.

Place the sample on the sample tray and set it at the same tray rail number that has been adjusted to the focus.

Shut the door of the dark box.

On the computer, select the Operation Mode and the necessary settings for conducting exposure.

3. Exposing Fluorescent Samples

Exposing Samples Using the Incident Light Method

Initial settings are same as those for exposure of chemiluminescent samples

Install a fluorescent lens filter by screwing it to the lens.

Place the sample on the tray and set it in the dark box.

Conduct exposure of a fluorescent screen

Save the fluorescent screen image.

Conduct exposure of fluorescent samples or enzyme amplification fluorescent samples.

2. Exposing Stained Gel or Film Samples

Exposing Samples by the Transmitted Light Method

Initial settings are same as those for exposure of chemiluminescent samples

Select whether or not to mount the filter, depending on the object. To conduct exposure of film or stained gel by the transmitted light method, on fluorescent lens filters are required.

Conduct Flat-Frame exposure without placing the sample on the sample tray for transmitting.

Set the sample tray for transmitting on the rail same as the sample that will be subjected to exposure.

Set the LED switch in the dark box down to turn the transmitted light LED ON.

Shut the door firmly until the click sounds. Change the image reading software mode to precision, the image-type to Flat-Frame, and then conduct exposure.

Confirm that the density of the image that has undergone exposure is within the range of 5,000 to 10,000.

Save the image file with appropriate name.

Place the same on the sample on tray for transmitting. Set it in the dar box, and then adjust the focus.

Set the LED switch in the dark box down to turn the transmitted light LED ON.

Change the image type to image and select Fluorescence in the Flat-Frame Correction on the right side.

3. Quitting the Image Reader LAS-1000

5.1 Quit the image reading software, Image Reader LAS-1000.

5.2. Turn OFF the power to the computer and turn off the power to the camera controller and the computer's peripheral devices.

4. Quantifying an Image by Image Gauge Ver. 2.54.

There are four analysis modes in this software: Annotation Mode, Quant Mode, Profile/MW Mode, and Distance. The Quant Mode was used in this study.

Quant Mode: Measures the radioactivity within a specified region. The mouse is used to enclose the region for measurement, and the radiation dose within that

region is displayed luminescence (PSL) in the standard. This mode can be used to apply background substraction, setting of standard values, and group formation, etc., to quantify the results.

6.1. Quant Mode---- Rectangle Tool

6.1.1. Select the Rectangle tool from the tool box in Quant mode.

6.1.2. Place the cursor on the edge of the desired region and drag it to enclose the region.

6.1.3. When the region has been appropriately enclosed, release the button on the mouse.

6.2. Analysis in Quant Mode

6.2.1. Select Quant Results from the Window menu. Quantification results will be displayed.

6.2.2. Select the items you want to have displayed when measurement results are displayed.

6.2.3. A Quant List Item dialog will be shown. By clicking the checked boxes to choose what data you want. In this study, the PSL data were chosed.

6.2.4. In put data to a excel sheet and graph data.

APPENDIX 4: Primers used in this study

	5'RACE primers	3'RACE primers	Full length cDNAs		Expression pattern		Overexpression	
Access No.	BE123605(SPT1) CO200178(SPT2	DR024381(SPT1) CO200178(SPT2)	5'fragment Forward	3'fragment Reverse	Full length		Full length	
Pt-SPT1	5'-TGGGTCCCTGAAGCCACTACATCCA-3'	5'TGCTGGTATGGGGTATGCTTTGGCA3'	5'-TCCCGCAGCACACCCTTGACCA-3'	5'-TTGCCTGACCTAGCTGAACATGAATGA-3'	5'CTAGCTAGCAAAGGTTCTGGACCTGGACTTATGAGA-3'	5'-CCGCTCGAGCCAAACTTTCAATCAACCCATACACCA-3'		
Pt-SPT2	5'-TTGCCGTTTTTCAGCACAACACACC-3'	5'-TGCAGGAAAGGCAACTGTCACCACTGC-3'	5'-CGAATGCGTGCAAGCTTTTGAGCTCCT-3'	5'-CGCCACATCAAAATGTGGTGCCATTC-3'	5'-GGGAATTCCATATGGGGGGCTTAAGTTGCGGTATTCGAT-3'	5'-CCGGAATTCGGCATGGTGCCAGTCAAGCCAGTA-3'		
Access no.	TC45250	TC45250	5'fragment Forward	3'fragment Reverse				
Pt-CERKs (Long version and short version)	5'-GAACCCCCACTAGCAAGCTCCACAGCA-3	5'-CCAATCCGAGATCGGGATGTGAT-3	5'-AGAGGGCGGGGACGTCCATCCA-3'	5'-TGTGCGCTTCCCCTCCCAATA-3'	5'-CTAGCTAGCTTCGAAGGTGGCGTTCTGAGCAACAA-3'	5'-CCGGAATTCTTGGAGGTGACCCCGGATTTAAACA-3'	5'-CCGGAATTCTTGGAGGTGACCCCGGATTTAAACA-3'	5'-CTAGCTAGCTTCGAAGGTAACGTTCTGAGCAACAA3
					5'-GAAGTACCCAATCCGAGATCGGGATGTGAT	5'-TCGACGAAAAACCTCGCCGTCCAC-3'	5'-TCGACGAAAAACCTCGCCGTCCAC-3'	5'-CTAGCTAGCGAGGGGGAAAGACCCCCGTCTAA-3'
Pt-CERKF								

APPENDIX 5: Alternative splicing results for SPT1 in human, Arabidopsis, and drosophila

A4.1. Human SPT1 alternative splicing results

>gi|46329879|gb|BC068537.1| Homo sapiens serine palmitoyltransferase, long chain base subunit 1, mRNA (cDNA clone MGC: 87384 IMAGE:5259635), complete cds

GGCTAACTATGGCGACCGCCACGGAGCAGTGGGTTCTGGTGGAGATGGTACAGGCGCTTTACGAGGCTCC
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AAATTACAAGAACGATCTGATCTTACAGTCAAGGAAAAAGAAGAACTGATTGAAGAGTGGCAACCAGAAC
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AAAAAAAAAAAA

>gi|2564246|emb|Y08685.1| H.sapiens mRNA for serine palmitoyltransferase, subunit I
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CCTCCTGTCCCAAAAGACCATCCTGCTCTCAACTACAACATCGTTTCAGGCCCTCCAAGCCACAAAACTG
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AAAAAAAAAAAAAAAAA

>gi|13937947:14-445 Homo sapiens serine palmitoyltransferase, long chain base subunit 1, mRNA (cDNA clone IMAGE:4094854), complete cds
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>gi|113426464:1-195 PREDICTED: Homo sapiens similar to serine palmitoyltransferase subunit 1 isoform b (LOC731770), mRNA
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1. Chromosome 9 (NC000009) with mRNA (BC068537.1) AAH68537

	Genomic coordinates	mRNA coordinates	length	identity	Mis-matches	gaps	Donor site
Exon 1	94703-94767	Jan-65	65	100%	0	0	d
Exon 2	91844-91951	66-173	108	100%	0	0	d
Exon 3	88129-88223	174-268	95	100%	0	0	d
Exon 4	60259-60352	269-362	94	100%	0	0	d
Exon 5	59405-59477	363-435	73	100%	0	0	d
Exon 6	47355-47487	436-568	133	100%	0	0	d
Exon 7	38568-38697	569-698	130	100%	0	0	d
Exon 8	34794-34883	699-788	90	100%	0	0	d
Exon 9	29349-29456	789-896	108	100%	0	0	d
Exon 10	27002-27097	897-992	96	100%	0	0	d
Exon 11	26561-26657	993-1089	97	100%	0	0	d
Exon 12	25388-25442	1090-1144	55	100%	0	0	d
Exon 13	17637-17754	1145-1262	118	100%	0	0	d
Exon 14	14199-14272	1263-1336	74	99%	1	0	d
Exon 15	10527-11915	1337-2725	1389	100%	0	0	

2. Chromosoms 9 (NC000009) with mRNA (Y08685.1)

	Genomic coordinates	mRNA coordinates	length	identity	Mis-matches	gaps	Donor site
Exon 1	94703-94759	Jan-57	57	100%	0	0	d
Exon 2	91844-91951	58-165	108	100%	0	0	d
Exon 3	88129-88223	166-260	95	100%	0	0	d
Exon 4	60259-60352	261-354	94	100%	0	0	d
Exon 5	59405-59477	355-427	73	100%	0	0	d
Exon 6	47355-47487	428-560	133	100%	0	0	d
Exon 7	38568-38697	561-690	130	100%	0	0	d
Exon 8	34794-34883	691-780	90	100%	0	0	d
Exon 9	29349-29456	781-888	108	100%	0	0	d
Exon 10	27002-27097	889-984	96	100%	0	0	d
Exon 11	26561-26657	985-1081	97	100%	0	0	d
Exon 12	25388-25442	1082-1136	55	100%	0	0	d
Exon 13	17637-17754	1137-1254	118	100%	0	0	d
Exon 14	14199-14272	1255-1328	74	99%	0	0	d
Exon 15	11753-11947	1329-1526	198	100%	6		

3. Chromosome 9 (NC000009) with NM_006415 2,780

	Genomic coordinates	mRNA coordinates	length	identity	Mis-matches	gaps	Donor site
Exon 1	94703-94797	Jan-95	95	100%	0	0	d
Exon 2	91844-91951	96-203	108	100%	0	0	d
Exon 3	88129-88223	204-298	95	100%	0	0	d
Exon 4	60259-60352	299-392	94	100%	0	0	d
Exon 5	59405-59477	393-465	73	100%	0	0	d
Exon 6	47355-47487	466-598	133	100%	0	0	d
Exon 7	38568-38697	599-728	130	100%	0	0	d
Exon 8	34794-34883	729-818	90	100%	0	0	d
Exon 9	29349-29456	819-926	108	100%	0	0	d
Exon 10	27002-27097	927-1022	96	100%	0	0	d
Exon 11	26561-26657	1023-1119	97	100%	0	0	d
Exon 12	25388-25442	1120-1174	55	100%	0	0	d
Exon 13	17637-17754	1175-1292	118	100%	0	0	d
Exon 14	14199-14272	1293-1366	74	99%	0	0	d
Exon 15	10534-11947	1367-2780	1414	100%	0	0	

4. Chromosome 9 (NC000009) and NM_178324

	Genomic coordinates	mRNA coordinates	length	identity	Mis-matches	gaps	Donor site
Exon 1	94703-94797	Jan-95	95	100%	0	0	d
Exon 2	91844-91951	96-203	108	100%	0	0	d
Exon 3	88129-88223	204-298	95	100%	0	0	d
Exon 4	60259-60352	299-392	94	100%	0	0	d
Exon 5	59405-59477	393-465	73	100%	0	0	d
Exon 6	58453-58955	466-968	503	100%	0	0	

5. Chromosome 9 with 13937947 (998bps)

	Genomic coordinates	mRNA coordinates	length	identity	Mis-matches	gaps	Donor site
Exon 1	94703-94759	Jan-57	57	100%	0	0	d
Exon 2	91844-91951	58-165	108	100%	0	0	d
Exon 3	88129-88223	166-260	95	100%	0	0	d
Exon 4	60259-60352	261-354	94	100%	0	0	d
Exon 5	59400-59477	355-432	78	100%	5	0	

6. Chromosome 9 with 113426464 (973bps)

	Genomic coordinates	mRNA coordinates	length	identity	Mis-matches	gaps	Donor site
Exon 1	94703-94759	Jan-57	57	100%	2	0	d
Exon 2	91844-91951	58-165	108	100%	3	0	d
Exon 3	88194-88223	166-195	30	100%	1	0	

A4.2. Arabidopsis alternative splicing results

>Chromosome_4_in_Arabidopsis_(_SPT1)
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serine palmitoyltransferase, complete cds

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thaliana DNA chromosome 4, contig fragment No. 85

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TACCTTGCAAGTGCTGCTATCACCGCCATTGATGTCATTGATCAAAACCCTGATATAAATGCTGGGAACA
ACGCAGGATTGTCAGATATCAAGGGGATGTCATTAACAAGCAACCGGGAATCACCTATTGTTTTCTTAAA
ATTAGAGAAATCTAGCGGTTCAGCTAAAGATGATTTGCTTCTACTTGAGAAAATGGCTGACCGTGCTCTG
AAGGAAGACTCATTGTTGGTTGTGAGTTCTAAAAGATCATTTCTTGATAAATGCCGGTTACCTGTGGGAA
TCAAACTCTACGTCTCAGCGGGACATTCGGAATCCGATCTTCTTAAAGCGTCGGAGTCACTGAAGAGACT
TGCTTCAGAGCTTCTACTCAAGTCCTGA

1. Chromosome 4 with At-SPT1(NM_001036719)

	Genomic coordinates	mRNA coordinates	length	identity	Mis-matches	gaps
Exon 1	1085-1198	88-201	114	93%	8	0
Exon 2	1323-1414	202-293	92	100%	0	0
Exon 3	1495-1599	294-398	105	95%	5	0
Exon 4	1678-1730	399-451	53	100%	0	0
Exon 5	1825-1951	452-578	127	96%	5	0
Exon 6	2090-2279	579-768	190	94%	11	0
Exon 7	2381-2521	769-909	141	100%	0	0
Exon 8	2608-2703	910-1005	96	100%	0	0
Exon 9	2796-2937	1006-1147	142	100%	0	0
Exon 10	3037-3164	1148-1275	128	100%	0	0
Exon 11	3239-3412	1276-1449	174	100%	0	0

2. Chromosome 4 with AB063254

	Genomic coordinates	mRNA coordinates	length	identity	Mis-matches	gaps	Donor site
Exon 1	865-991	1-127	127	100%	0	0	d
Exon 2	1104-1198	128-222	95	100%	0	0	d
Exon 3	1323-1414	223-314	92	100%	0	0	d
Exon 4	1495-1599	315-419	105	95%	5	0	d
Exon 5	1678-1730	420-472	53	100%	0	0	d
Exon 6	1825-1951	473-599	127	96%	5	0	d
Exon 7	2090-2279	600-789	190	94%	11	0	d
Exon 8	2381-2521	790-930	141	100%	0	0	d
Exon 9	2608-2703	931-1026	96	100%	0	0	d
Exon 10	2796-2937	1027-1168	142	100%	0	0	d
Exon 11	3037-3164	1169-1296	128	100%	0	0	d
Exon 12	3239-3755	1297-1813	517	100%	0	0	

3. Chromosome4 with AY120759

	Genomic coordinates	mRNA coordinates	length	identity	Mis-matches	gaps	Donor site
Exon 1	379-575	1-197	197	100%	0	0	d
Exon 2	786-991	198-403	206	100%	0	0	d
Exon 3	1104-1198	404-498	95	100%	0	0	d
Exon 4	1323-1414	499-590	92	100%	0	0	d
Exon 5	1495-1599	591-695	105	95%	5	0	d
Exon 6	1678-1730	696-748	53	100%	0	0	d
Exon 7	1825-1951	749-875	127	96%	5	0	d
Exon 8	2090-2279	876-1065	190	94%	11	0	d
Exon 9	2381-2521	1066-1206	141	100%	0	0	d
Exon 10	2608-2703	1207-1302	96	100%	0	0	d
Exon 11	2796-2937	1303-1444	142	100%	0	0	d
Exon 12	3037-3164	1445-1572	128	100%	0	0	d
Exon 13	3239-3764	1573-2098	526	100%	0	0	

4. Chromosome4 with AAN15450

	Genomic coordinates	mRNA coordinates	length	identity	Mis-matches	gaps	Donor site
Exon 1	1085-1198	88-201	114	93%	8	0	d
Exon 2	1323-1414	202-293	92	100%	0	0	d
Exon 3	1495-1599	294-398	105	95%	5	0	d
Exon 4	1678-1730	399-451	53	100%	0	0	d
Exon 5	1825-1951	452-578	127	96%	5	0	d
Exon 6	2090-2279	579-768	190	94%	11	0	d
Exon 7	2381-2521	769-909	141	100%	0	0	d
Exon 8	2608-2703	910-1005	96	100%	0	0	d
Exon 9	2796-2937	1006-1147	142	100%	0	0	d
Exon 10	3037-3164	1148-1275	128	100%	0	0	d
Exon 11	3239-3412	1276-1449	174	100%	0	0	

5. Chromosome 4 with CAB80314

	Genomic coordinates	mRNA coordinates	length	identity	Mis-matches	gaps	Donor site
Exon 1	1085-1198	88-201	114	93%	8	0	d
Exon 2	1323-1414	202-293	92	100%	0	0	d
Exon 3	1495-1599	294-398	105	95%	5	0	d
Exon 4	1678-1730	399-451	53	100%	0	0	d
Exon 5	1825-1951	452-578	127	96%	5	0	d
Exon 6	2090-2279	579-768	190	94%	11	0	d
Exon 7	2381-2521	769-909	141	100%	0	0	d
Exon 8	2608-2703	910-1005	96	100%	0	0	d
Exon 9	2796-2896	1006-1106	101	100%	0	0	d
Exon 10	3017-3164	1107-1254	148	100%	0	0	d
Exon 11	3239-3412	1255-1428	174	100%	0	0	

A4.3. Alternative splicing results in drosophila

NP_725256:

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>gi|28573408:98-1504 Drosophila melanogaster Serine
palmitoyltransferase subunit I CG4016-RC, transcript variant C (Spt-I),
mRNA
ATGGTGGCCATCCAATTGTTCAACGAAATCGGCAGTATTTTCCGAAACACCCCCACGTTTGCTCTCGTCT
TGGAGACCCTACTGCTCATAACCGTGATTTGGTTGCTGCTCCACAGACGAGGAGGAGGTCGTCGCCGACA
GCTGACCAAGGAGGAGGAAGACCGAATAATCGCCGACTATGAACCAGAGCCCTTGGTGGCCGACACCGAT
CCCAATCATCCCCTGCTGCACACCCGCGTTGTGCAGTCCAGGGTGGGCAAGCGCATCCAGGTCGATGGAC
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ACGACTGCCTAAATCTTGGCTCTCACAACTATCTTGGCTTCCTCGAGGACCAGGAGATACTGGAGGAGGC
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CATCTGGACCTGGAGGATCGTATTGCCAAGTTCATGGGCCTGGAGGAGGCCATTGTCTATTCCTACGGCT
TCTCGACCGTGGCCAGTGCCATTCCGGCGTATGCCAAACGTGGCGACCTTATCTTTGTGGATGAGGCTGT
CAACTTTGCCATTCAGAAGGGTCTGGATGCTTCACGAAGCACAATCGTTTTCTTTAAGCACAATGACGTT
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CACGTCGCTTCCTCGTCGCCGAGGGTATCTATATGAATACGGGCGAGATTTGCCCACTTCCCGACCTGGT
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GGTGGTCACGGAGTTACGGAGCACTTTAATGTCGATCGTGATGAAGTCGATCTGATATCGGCCGGCATGG
AGGGTTCGATGGCAACAGTCGGTGGCTTCTGTGTTGGCTCGCACTTCATAGCCGAGCACCAGCGCCTCTC
TGGCTTGGGCTACATCTTCTCGGCCTCGCTGCCGCCCATGCTCACCCAGGCGGCTATTTCAGCGTTGGAT
CGTTTCGAACGAGAACCACAAATCTTCGAGCAGCTGCAGGCAAAGTCTAAGACGTTGCACCAGAAGTTTT
TGCGATTCAGCAAGCTAACCCTGCGCGGCGATGAGGTGTCACCAGTGAAGCACTTGTATCTTGCCCAGCC
CGCCGAGAACTTTGACAAGGAACTGAAACTTCTAACAGAGCTGGCTGATAAGTGCATTGCTCGTGGAGTG
GCTGTGGTACAGGCTGCCTACCTGCAGAACAGGGAACGCCAACCAGTCCGTCCCAGCATTCGCATTGCTG
TCAACCGTTTGCTGGAGAGTTCGGAAATAGACAATGCGTTTGAGGTCATCGAGAGTGTTTCCAGCTCCGT
CCTATAA

>gi|28573407|ref|NM_165967.2| Drosophila melanogaster Serine palmitoyltransferase subunit I CG4016-RB, transcript variant B (Spt-I), mRNA
AGTACATTCGTTCATCCATCTGTTTCGGCGGATATTTCACGCATTTTGCCTATAGTTAACTGAGTTTTGT
GGTGAGTGCTCGATTTAAATGGATATTTAATAAAGGGCACGGAACCAGACTGTCAAAATGGTGGCCATCC
AATTGTTCAACGAAATCGGCAGTATTTTCCGAAACACCCCCACGTTTGCTCTCGTCTTGGAGACCCTACT
GCTCATAACCGTGATTTGGTTGCTGCTCCACAGACGAGGAGGAGGTCGTCGCCGACAGCTGACCAAGGAG
GAGGAAGACCGAATAATCGCCGACTATGAACCAGAGCCCTTGGTGGCCGACACCGATCCCAATCATCCCC
TGCTGCACACCCGCGTTGTGCAGTCCAGGGTGGGCAAGCGCATCCAGGTCGATGGACACGACTGCCTAAA
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AGGATCGTATTGCCAAGTTCATGGGCCTGGAGGAGGCCATTGTCTATTCCTACGGCTTCTCGACCGTGGC
CAGTGCCATTCCGGCGTATGCCAAACGTGGCGACCTTATCTTTGTGGATGAGGCTGTCAACTTTGCCATT
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GTCTGCTGATTGAGCAAGAAAAACGAGACCAGAAAAATCCGAAAAAGGCGGCTAAGACACGTCGCTTCCT
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TTACGGAGCACTTTAATGTCGATCGTGATGAAGTCGATCTGATATCGGCCGGCATGGAGGGTTCGATGGC
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GACAAGGAACTGAAACTTCTAACAGAGCTGGCTGATAAGTGCATTGCTCGTGGAGTGGCTGTGGTACAGG
CTGCCTACCTGCAGAACAGGGAACGCCAACCAGTCCGTCCCAGCATTCGCATTGCTGTCAACCGTTTGCT
GGAGAGTTCGGAAATAGACAATGCGTTTGAGGTCATCGAGAGTGTTTCCAGCTCCGTCCTATAAGCCTTG
TTGTTGGAAAGGAATGATCTTCTTTCGACAATGGCCTTGCTGTTAAGTTTT

>gi|24653275|ref|NM_136998.1| Drosophila melanogaster Serine palmitoyltransferase subunit I CG4016-RA, transcript variant A (Spt-I), mRNA
AATTAAGGCGGTTTCAATTGATTTTTCACAGAAAGGGCACGGAACCAGACTGTCAAAATGGTGGCCATCC
AATTGTTCAACGAAATCGGCAGTATTTTCCGAAACACCCCCACGTTTGCTCTCGTCTTGGAGACCCTACT
GCTCATAACCGTGATTTGGTTGCTGCTCCACAGACGAGGAGGAGGTCGTCGCCGACAGCTGACCAAGGAG
GAGGAAGACCGAATAATCGCCGACTATGAACCAGAGCCCTTGGTGGCCGACACCGATCCCAATCATCCCC
TGCTGCACACCCGCGTTGTGCAGTCCAGGGTGGGCAAGCGCATCCAGGTCGATGGACACGACTGCCTAAA
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CGCAAGTACGGAGTTGGATCTTGCGGACCTCGGGGCTTCTACGGCACTATGGACGTGCATCTGGACCTGG
AGGATCGTATTGCCAAGTTCATGGGCCTGGAGGAGGCCATTGTCTATTCCTACGGCTTCTCGACCGTGGC
CAGTGCCATTCCGGCGTATGCCAAACGTGGCGACCTTATCTTTGTGGATGAGGCTGTCAACTTTGCCATT

CAGAAGGGTCTGGATGCTTCACGAAGCACAATCGTTTTCTTTAAGCACAATGACGTTGAGGATCTAGAAC
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CGTCGCCGAGGGTATCTATATGAATACGGGCGAGATTTGCCCACTTCCCGACCTGGTGGCCTTGCGTCAG
AAGTACAAGCTGCGTTTGTTCATCGACGAAAGCATCTCCTTTGGCACATTGGGCCAGGGTGGTCACGGAG
TTACGGAGCACTTTAATGTCGATCGTGATGAAGTCGATCTGATATCGGCCGGCATGGAGGGTTCGATGGC
AACAGTCGGTGGCTTCTGTGTTGGCTCGCACTTCATAGCCGAGCACCAGCGCCTCTCTGGCTTGGGCTAC
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GCTAACCCTGCGCGGCGATGAGGTGTCACCAGTGAAGCACTTGTATCTTGCCCAGCCCGCCGAGAACTTT
GACAAGGAACTGAAACTTCTAACAGAGCTGGCTGATAAGTGCATTGCTCGTGGAGTGGCTGTGGTACAGG
CTGCCTACCTGCAGAACAGGGAACGCCAACCAGTCCGTCCCAGCATTCGCATTGCTGTCAACCGTTTGCT
GGAGAGTTCGGAAATAGACAATGCGTTTGAGGTCATCGAGAGTGTTTCCAGCTCCGTCCTATAAGCCTTG
TTGTTGGAAAGGAATGATCTTCTTTCGACAATGGCCTTGCTGTTAAGTTTT

1. Splicing results between NP_725256 and the chromosome 2R

	Genomic coordinates	mRNA coordinates	length	identity	Mis-matches	gaps	Donor site
Exon 1	597-644	Jan-48	48	100%	0	0	d
Exon 2	707-2011	49-1242	1194	91%	111	111	

2. Splicing results between NM_165967 and chromosome 2R

	Genomic coordinates	mRNA coordinates	length	identity	Mis-matches	gaps	Donor site
Exon 1	256-356	1-101	101	100%	0	0	d
Exon 2	571-644	102-175	74	100%	0	0	d
Exon 3	707-2011	176-1369	1194	91%	111	111	

3. Splicing results between NM_033778 and chromosome 2R

	Genomic coordinates	mRNA coordinates	length	identity	Mis-matches	gaps	Donor site
Exon 1	540-644	1-105	105	100%	0	0	d
Exon 2	707-2011	106-1299	1194	91%	111	111	

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VITA

Cuihua Zhu was born in China and got her Bachelor degree in forestry protection in 1991 from Nanjing Forestry University. After graduation, she worked in China National Tree Seed Corporation as a research scientist to establish the seed and seedling control lab and conduct research in seed quality testing, seed dormancy broken, seed disease control for about nine years. There she has improved the international test methods for about 206 wild species. At the same time, she has been in charge of the seed and seedling quarantine and handled some seed and seedling export and import around the world. She came to the United Stated and started her graduate study in Auburn University in 2001 and got her Master degree in 2003. In the fall of 2003, she joined Dr. Cairney's lab and worked as a technician to conduct research to investigate the roles of sphingolipids on Loblolly pine. In 2004, she started her Ph.D study under Dr. Cairney's guidance. Her dissertation research focuses on the roles of sphingolipids on embryogenesis of Loblolly Pine. Over the period of four years of Ph.D study, she cloned a few genes, which encoded the two types of key enzymes in sphingolipid metabolism and proved that those enzymes have conserved metallic functions same to their homologs in Arabidopsis and mammals.